FORMULATION AND DEVELOPMENT OF CATIONIC LIPOSOMES AS ADJUVANTS FOR SUBUNIT PROTEIN VACCINES

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Summary

Liposomes remain at the forefront of vaccine design due to their well documented abilities to act as delivery vehicles and adjuvants. Liposomes have been described to initiate an antigen depot-effect, thereby increasing antigen exposure to circulating antigen-presenting cells. More recently, in-depth reviews have focussed on inherent immunostimulatory abilities of various cationic lipids, the use of which is consequently of interest in the development of subunit protein vaccines which when delivered without an adjuvant are poorly immunogenic.

The importance of liposomes for the mediation of an antigen depot-effect was examined by use of a dual-radiolabelling technique thereby allowing simultaneous detection of liposomal and antigenic components and analysis of their pharmacokinetic profile. In addition to investigating the biodistribution of these formulations, their physicochemical properties were analysed and the ability of the various liposome formulations to elicit humoral and cell-mediated immune responses was investigated.

Our results show a requirement of cationic charge and medium/strong levels of antigen adsorption to the cationic liposome in order for both a liposome and antigen depot-effect to occur at the injection site. The choice of injection route had little effect on the pharmacokinetics or immunogenicity observed. *In vitro*, cationic liposomes were more cytotoxic than neutral liposomes due to significantly enhanced levels of cell uptake. With regards to the role of bilayer fluidity, liposomes expressing more rigid bilayers displayed increased retention at the injection site although this did not necessarily result in increased antigen retention. Furthermore, liposome bilayer rigidity did not necessarily correlate with improved immunogenicity. In similar findings, liposome size did not appear to control liposome or antigen retention at the injection site. However, a strong liposome size correlation between splenocyte proliferation and production of IL-10 was noted; specifically immunisation with large liposomes lead to increased levels of splenocyte proliferation coupled with decreased IL-10 production.

Key words: delivery vesicle, depot-effect, biodistribution, tuberculosis, immune response

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In science credit goes to the man who convinces the world, not the man to whom the idea first occurs.

—Francis Galton

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Abbreviations

APC	Antigen presenting cells
BCA	Bicinchoninic acid protein assay
BLN	Brachial lymph node
BSA	Bovine serum albumin
Chol	Cholesterol
ConA	Concanavalin A
CTH1	CtHybrid1
DAMP	Damage associated molecular patterns
DC	Dendritic cell
DC-Chol	3β-[N-(N',N'-dimethylaminoethane)-carbomyl] cholesterol
DDA	Dimethyldioctadecylammonium bromide
DMSO	Dimethyl sulphoxide
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DSPC	Distearoyl-glycero-phosphatidylcholine
ELISA	Enzyme-linked immunosorbent assay
H1	Ag85B-ESAT-6
ICF	Incomplete Freunds's adjuvant
i.d	Intradermal
i.m	Intramuscular
i.ln	Intralymphatic
LYZ	Lysozyme
MDP	*N*-Acetylmuramyl-L-alanyl-D-isoglutamine; muramyl dipeptide
MHC ClI/II	Major histocompatibility complex class I/II
MPL	Monophosphoryl lipid A
NR	Neutral red
OVA	Ovalbumin
PAMP	Pathogen associated molecular patterns
p.f.i	post final injection
p.i	post injection
pI	Isoelectric point
PLN	Popliteal lymph node
PRR	Pattern recognition receptors

rbf	Round bottomed flask
Rho-DPPE	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt)
s.c	Subcutaneous
TB	Tuberculosis
TCR	T cell receptor
TDB	Trehalose 6,6'-dibehenate
TDM	Trehalose 6,6'-dimycolate
TLR	Toll like receptor
ZP	Zeta potential

Chapter 1: General Introduction

1.1 The History of Vaccination

The origins of vaccination lie with smallpox, a disease which once ravaged most, if not all parts of the world. Initial descriptions of smallpox stem from as early as 1122 BC from texts originating in China. Smallpox was known to spread rapidly and resulted in disfigurement, blindness and death. It was also known that smallpox was infectious and early reports dating from 430 BC describe survivors of smallpox being used to treat those infected in a process known as inoculation (Gross and Sepkowitz, 1998). Over many hundreds of years the concept of inoculation began to take form and involved a small swab of infectious material (otherwise known as pus) being placed on the skin of non-infected persons. However, it was not until 1722, when members of the English Royal Family were successfully immunised against smallpox using this method, that inoculation was accepted in the scientific community of the western-world (Riedel, 2005). For the following 70 years many people were inoculated against smallpox using infected material derived from those infected with smallpox. One such person was Edward Jenner, born in 1749 in Gloucestershire, UK. He developed a strong interest in science and after overhearing a young dairymaid claim that she may never have smallpox as she had had cowpox, he decided to investigate further. In 1796 Jenner successfully inoculated a young boy with infectious material from a dairymaid who had cowpox lesions; two months later he exposed the boy to smallpox and no disease resulted. This was the beginning of what we now term vaccination.

1.2 Current Vaccination Campaigns with Focus on Tuberculosis

The process of vaccination is now commonplace in the western world where drugs and medical treatment are relatively accessible. Whilst vaccination is of principle benefit to the individual receiving the vaccine, the concept of herd immunity is possibly more important as vaccination campaigns are dependent on the development of herd immunity to successfully eradicate disease. This was classically seen in 1980 when the world was declared free of smallpox and likewise plans have played a primary role in the success of diminishing polio cases. The World Health Organisation (WHO) campaign to eradicate polio has however been blighted by political instabilities with consequential poor vaccine uptake and poor herd immunity. To date, polio remains endemic in four countries (India, Pakistan, Afghanistan and Nigeria) and in 2009 there were just 1604 cases worldwide, a far cry from the 350,000 cases in 1988 when the worldwide vaccination campaign began (The

Global Polio Eradication Initiative, 2010). In addition to polio, elimination of the diseases measles and tetanus by vaccination is also being actively supported by the WHO (The World Heath Organisation, 2009).

We have now entered 'the decade of vaccines' (The Lancet Infectious Diseases, 2010) in which many of the obstacles faced in bringing vaccines from the laboratory to the clinic will hopefully be addressed. Possibly the most important aspect is funding for the discovery and development of vaccines, however the financial gain of vaccine production to companies obviously remains an important issue. In 2008 the vaccine market was estimated to be worth €11.5 billion and substantial expansion is predicted (Taylor et al., 2009), especially from developing country manufactures (Milstien and Kaddar, 2010). However, unlike drug development, vaccine development does not offer the same financial gain, especially for the development of vaccines against diseases of the developing world. Approaches such as those applied by GlaxoSmithKline (GSK) Biologicals in collaboration with The Global Alliance for Vaccines and Immunisation (GAVI) have resulted in the provision of vaccines to the world's poorest countries at prices typically 10 - 25 % of those paid by the developed world (Taylor et al., 2009). Strategies such as these are the only way in which vaccination can be offered to everyone and therefore help in the worldwide aim to eradicate particularly infectious and debilitating diseases.

With regards to funding of vaccine research and development, there still remains a significant lack of cash inflow for diseases of the developing world, aptly termed neglected diseases. With the celebration of GAVI's 10th birthday also arrived a $10 billion payment from the Bill and Melinda Gates foundation, a sum which will significantly help in the distribution of the vaccines it disburses against diphtheria, tetanus, pertussis, hepatitis B and *Haemophilus influenzae* type b (The Lancet Infectious Diseases, 2010). However, $10 billion represents just a small amount when one considers that an estimated $2 billion is needed every year for the continuing discovery and development of a vaccine against tuberculosis (TB) alone (Kaufmann et al., 2010). Although the current TB vaccine funding is significantly lower than required, the first decade of the millennia has still been an relatively productive period (Table 1.1); in 2009 there were 10 vaccines undergoing clinical trials with a further 6 in preclinical studies and over 22 candidate formulations (Stop TB Partnership, 2009). Interestingly, and discussed in detail later, there remains huge scope in the type of vaccine, proposed vaccine schedule and antigen/adjuvant selected, all of which

further highlight the complexity of designing vaccines against TB and other diseases (Kaufmann et al., 2010).

Underlined components are antigens requiring adjuvants. Data collected from (Stop TB Partnership, 2009).

The current vaccination campaign in the UK recommends vaccination against the diphtheria, measles, mumps, rubella, tetanus, polio, *Haemophilus influenzae* type b (Hib), pneumococcal, meningitis C and human papillomavirus types 16 and 18. Non-routine vaccines include the Bacillus Calmette-Guérin (BCG) and hepatitis B vaccines for those who are high-risk individuals (National Health Service, 2010). Between 1953 and 2005 it was national policy to vaccinate against TB using the BCG vaccine. However, the BCG vaccine was removed from the recommended immunisation schedule in the UK as it was no longer cost effective in a country which saw so few cases of TB. Furthermore, side-effects of the vaccine such as abscesses outweighed the preventative role of the vaccine. Importantly, numerous studies have shown huge variance in the efficacy of the BCG vaccine with values ranging from 0 – 80 % quoted. With regards to this, it is surprising that the BCG vaccine remains the most commonly used vaccine worldwide with over 3 billion people having received it (Andersen and Doherty, 2005). Although very successful as a vaccine to prevent

TB in newborn infants, the pitfalls of the BCG include its poor ability to maintain long term immunity and poor efficiency at preventing establishment of latent TB (Andersen, 2007). The most recent report on the number of TB cases in the UK shows the incidence of TB to be increasing (Moore et al., 2009). In 2008 there were 8,655 cases of TB diagnosed, equivalent to an increase of 2.9 % in the number of cases since 2007. The number of cases in the under 5 age group (which is often used as an indicator of recent transmission) has remained stable at approximately 5 in 100,000 since 2000 (Moore et al., 2009). However, an interesting finding in the demographics of TB incidence show that whilst the majority of cases in UK-born persons are in the 85 - 89 year age group (10 in 100,000), in non-UK born persons this figure is over 10-fold higher (123 in 100,000) and principally affected those between 25 - 29 years of age. Assuming that all UK-born persons received the BCG, this data would support the poor long-term immunising ability of the BCG vaccine with active TB posing a significant problem in immunocompromised ageing populations. In contrast, non-UK born persons show increased rates of TB at a young age when the immune system should be able to prevent onset of disease (assuming they had been vaccinated and it had been successful). Therefore with regards to tackling TB in the UK, there are two target groups: those who have previously been vaccinated against TB with a vaccine that does not confer life-long protection, and those that have not previously been vaccinated and act as a disease reservoir. The WHO estimates the number of new cases of TB in 2007 to be 9.27 million with a mortality of nearly 1.4 million (Bauquerez et al., 2009). In relation to global HIV incidence (2.7 million) in the same year, the scale of the TB pandemic is clear. It is now accepted that the previous long-term goal to reduce TB incidence to less than one case per million by 2050 (Dye et al., 2006) will now not be accomplished, and an intensified research vaccine development strategy with increases in funding is required (Kaufmann et al., 2010; Lönnroth et al., 2010). It should be noted that global vaccine research has intensified over the last 13 years with dramatic increases in the number of companies developing vaccines as well as the number of vaccines entering pre-clinical trials (Davis et al., 2010).

1.3 Overview of the Immune System

The immune system comprises many cellular and humoral components which must be able to protect the host from disease, a term which includes infection, autoimmune syndromes, injury and mutations. To do so, the host has acquired the ability to evolve to its environment, a classic example being the gut whereby commensal microbes live in

harmony with the host. The immune system can be divided into innate and acquired parts although interplay between the two is vital and there are many crossover components. The innate immune system can be described as the non-specific and rapid response to infection but the results are short lived and non-protective against future infection. In contrast, the acquired immune system is specific and long-term but equally takes time to establish. The aim of a vaccine is to mimic the immune response provoked by primary infection (hence the innate immune response) of a pathogenic substance but without causing the associated pathogenicity and eliciting significantly strong memory responses to protect the host upon future infection (hence the adaptive immune system). To do so both innate and acquired immune systems, comprising cellular and humoral components, must be stimulated in a process that involves recognition, uptake and presentation of antigen with subsequent establishment of a population of memory T and B cells (a concise review is given in (McCullough and Summerfield, 2005). The general processes involved in pathogen recognition through to antigen presentation to T cells are explained below.

1.3.1 Recognition of pathogenic material

Primary exposure to a pathogen results in a rapid non-specific response including the production of complement proteins and cytokines, both of which are capable of alerting circulating granulocytes (neutrophils, eosinophils and basophils) and mononuclear phagocytes (monocytes and macrophages) of a potential infection. The production of cytokines by granulocytes and mononuclear phagocytes results in local inflammation characterised by vasodilation, increased blood supply and initiation of a positive feedback loop with high amounts of pro-inflammatory cytokines, chemokines and phagocytes. Whilst local tissue damage plays an important role in the production of the humoral substances, pathogen derived factors such as toxins (e.g. from pathogens such as *Corynebacterium diphtheriae,* causing diphtheria, or *Clostridium tetani*, causing tetanus) or structural components such as bacterial flagella or surface glycoproteins may also initiate activation of the innate immune response. Pathogen association with host humoral (complement and antibodies) and cellular components initiates a series of events leading to pathogen uptake and consequent processing.

1.3.2 Mechanisms of uptake

Endocytosis is an umbrella term to describe the uptake of both host and pathogen derived macromolecules (Khalil et al., 2006). Endocytosis can be divided into phagocytosis (cell eating) and pinocytosis (cell drinking) depending on factors such as the size of the substance being uptaken, the receptors or ligands (if any) required and the components of the cells intracellular matrix (e.g. actin cytoskeleton) involved (Figure 1.1).

Figure 1.1 Mechanisms of endocytosis. Depicted here are phagocytosis used to clear dead cells and large pathogens (> 0.5 µm), macropinocytosis stimulated by mitogens to uptake pathogens of <5 µm, clathrin-mediated endocytosis involving uptake of small (~ 50 nm) pathogens and finally caveolae-mediate endocytosis which mediates uptake of pathogens in the size range of 100 - 150 nm. Only the latter mechanism does not result in vesicle-lysosome association with consequent degradation by lysosomal enzymes. Adapted from (Khalil et al., 2006).

Whilst it is generally considered that the consequence of endocytosis is lysosomal degradation of the pathogenic substance, as noted in Figure 1.1 this is not always the case as some intracellular vesicles are 'leaky' whilst others do not deliver their contents to lysosomes, instead targeting the endoplasmic reticulum and the Golgi apparatus (caveolae-mediated endocytosis). Larger (> 0.5 µm) pathogens are taken up by phagocytosis after binding with specific cell surface receptors such as Fcγ receptors. In this

case the pathogen must have encountered the opsonin system which comprises circulating antibodies and complement proteins that can bind pathogens. Macrophages are considered to be the professional phagocytic cell and are avid scavengers for both pathogenic substances and host infected cells expressing 'eat-me' signals (Hume, 2008). Pathogens of < 5 μm can be taken up by macropinocytosis or clathrin/caveolae mediated endocytosis (Khalil et al., 2006). For uptake routes in which endosome-lysosome fusion can occur, there is a decrease in pH as the endosomes matures. This feature has been used by pathogens such as *Listeria* and *Shigella* as a method to escape lysosomal degradation (Cossart and Roy, 2010).

It is postulated that such a variety of uptake mechanisms evolved to activate different downstream targets – knowledge of these different mechanisms is therefore of particular interest to vaccine development as it offers the possibility of selective targeting of specific immune responses depending on the antigen uptake route. The downstream target for the host is the processing and presentation of pathogen-derived antigens to T cells so that 'Memory' can be formed. Pathogens entering the cell via endocytosis are presented on MHC Cl II molecules whilst cytoplasmic pathogens are presented on MHC Cl I molecules. Whilst MHC Cl II molecules present antigens to CD4 expressing T cells (or Th cells), and MHC Cl I molecules present to CD8 expressing T cells (Tc cells), there is an additional mechanism known as cross-presentation whereby exogenous antigen can avoid MHC Cl II loading and instead be processed by proteasomes with subsequent MHC Cl I presentation. This mechanism has been attributed to DCs (Belz et al., 2002; den Haan and Bevan, 2001) and macrophages, albeit to a lesser ability (Trombetta and Mellman, 2005) and is of interest to vaccine design concerned with pathogens that do not directly infect APCs (Bhardwaj, 2001).

1.3.3 Antigen presentation and activation of T cells

T cells emerging from the thymic cortex and medulla are naïve until exposed to antigen and it is not until T cell receptor (TCR):antigen:MHC association with the relevant co-stimulatory molecules are T cells activated to become 'effector' T cells. T cells which have already encountered antigen can be successfully activated without the requirement of relevant co-stimulatory signals. Figure 1.2 outlines the different methods in which T and B cells become activated.

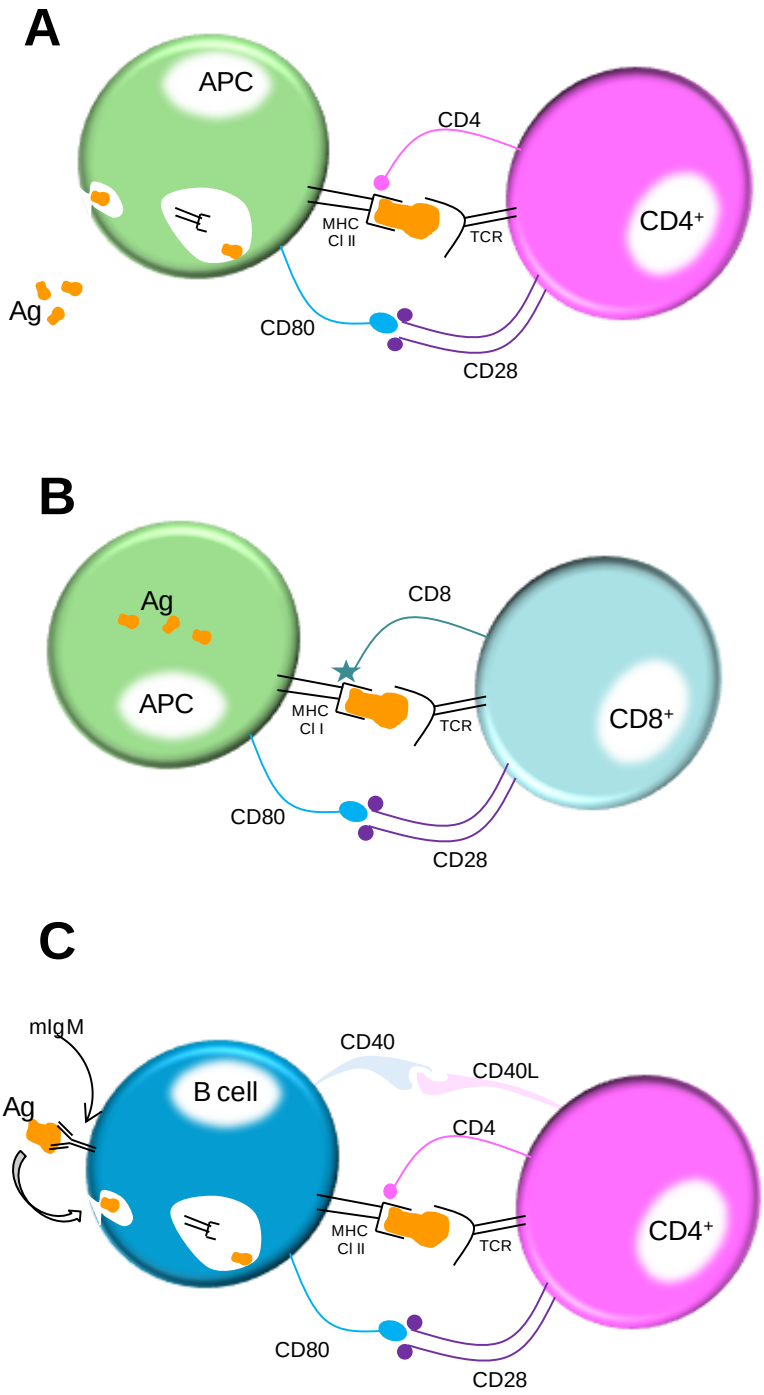

Figure 1.2 Mechanisms of T cell activation upon uptake and presentation of antigen (Ag) by APCs. A; Exogenous antigens are uptaken by APCs and processed for display on MHC Cl II molecules. $CD4^+$ T cells recognise antigen via their T cell receptor (TCR) and with co-stimulation from CD80-CD28 interactions, intracellular signalling pathways leading to $CD4^+$ T cell activation can occur. B; In a similar mechanism to 'A' but involving endogenous antigen, $CD8^+$ T cells can become activated by antigen presented on MHC Cl I molecules. C; B cells are also APCs however they require CD40-CD40L interactions in addition to CD80-CD28 for effective $CD4^+$ T cell activation to occur. Furthermore, antigen uptake is mediated by association with surface bound membrane IgM antibodies (mIgM).

Both MHC Cl II:$CD4^+$ T cell and MHC Cl I:$CD8^+$ T cell antigen presentation follows a similar mechanism: associated with the TCR are the relevant CD4 or CD8 molecules which are capable of binding protein tyrosine kinases (PTK), and the intracellular domains of the TCR itself contain ITAMs which can be phosphorylated. This TCR complex forms signal 1 of the activation process but for successful activation a co-stimulatory signal known as signal 2 is also required (lack of signal 2 leads to peripheral tolerance and anergy of the T cell whereby it is incapable of responding to self or non-self antigen). Binding of co-stimulatory molecules CD80 (on the APC) and CD28 (on the T cell) form signal 2, the strength of which is increased upon activation of signal 1. This immunological synapse is bought into play by adhesion molecules such as DC-SIGN, ICAM-3 and LFA-1, all of which rearrange to stabilise the clustering of TCR:MHC molecules.

Whilst DCs constitutively express CD80, macrophages and B cells must become activated for the expression of CD80. For this reason DCs are known as professional APCs however their phenotype is dependent on their location; within the peripheral tissues or the site of antigen uptake they are relatively poor at antigen presentation however upon their migration to the lymph nodes there is upregulation of co-stimulatory and MHC molecules so that in the lymph nodes they mature into potent APCs.

1.3.3.1 Activating intracellular pathways

The series of intracellular events that are initiated by association of the MHC:TCR synapse involves PTKs, autophosphorylation of ITAMs and activation of the intracellular PTK Zap70. Zap70 activates two routes, the first involving the molecule IP_3 and calcium release from the intracellular Ca^{2+} pool, and the second involving the signalling cascade DAG-PKC-CARMA1-Bcl10-MALT1 eventual NFκB mediated gene transcription (Ruland and Mak, 2003; Schmitz et al., 2003). Ca^{2+} acts as an activational secondary-signalling molecule and can induce exocytosis of granules acting as alarmins. Whilst this series of events relates to T cells during presentation of antigen, there are also numerous intracellular signalling pathways which occur in the APC which lead to upregulation of co-stimulatory molecules such as CD80 and CD40, upregulation of MHC molecules and production of chemokines, cytokines and other soluble factors which are all involved in the immune response. Initiation of such signalling pathways involves the binding of pathogenic components with numerous cellular receptors found on both myeloid and lymphoid cells. The best

described group of receptors are the Toll like receptors (TLRs) which are present on both the cell surface (TLRs 1, 2, 4, 5 and 6) and within the endosomal compartment (TLRs 3, 7, 8 and 9). TLRs are known to signal via intracellular MyD88 or Trif adapter proteins followed by activation of either the IRF3 or TRAF6 pathways; there are numerous in depth reviews focussing on TLR signalling pathways (see (Harding and Boom, 2010; Kawai and Akira, 2010; Monie et al., 2009)). Non-TLR receptors are also involved in pathogen recognition; these include C-type lectins such as Dectin-1, mannose, DC-SIGN and Mincle which signal via the CARD9-Bcl10-MALT10 pathway (Gregorio et al., 2009; Gross et al., 2006; Werninghaus et al., 2009). The role of these receptor-pathogen interactions with regards to vaccines and targeting the immune response are discussed in further detail in sections 1.4 and 1.5.

1.3.3.2 Localisation of responses

As mentioned briefly above, DCs only become efficient APCs upon entering the lymph nodes – in the periphery they do not express sufficient MHC or co-stimulatory molecules to successfully present antigen and activate T cells. Figure 1.3 shows a schematic of the lymph node. The lymph nodes, which drain circulating lymph from the tissues unlike the spleen which is involved in filtering blood-borne antigen, are placed strategically around the body to maximise their chances of encountering pathogens. The highly organised nature of the lymph node involves regions of APC:T cell interactions (the paracortex), areas of antibody production by plasma cells (the medulla) and the outermost 'cortex' layer in which memory B cells form (Figure 1.3). By having such an organisation it allows naïve T cell interactions with APCs to occur in the T cell rich paracortex area whilst effector T (and B) cells which are involved in secondary responses lie in the follicle rich cortex. Primary follicles form secondary follicles upon antigen exposure and are composed of germinal centres in which memory T and B cells form.

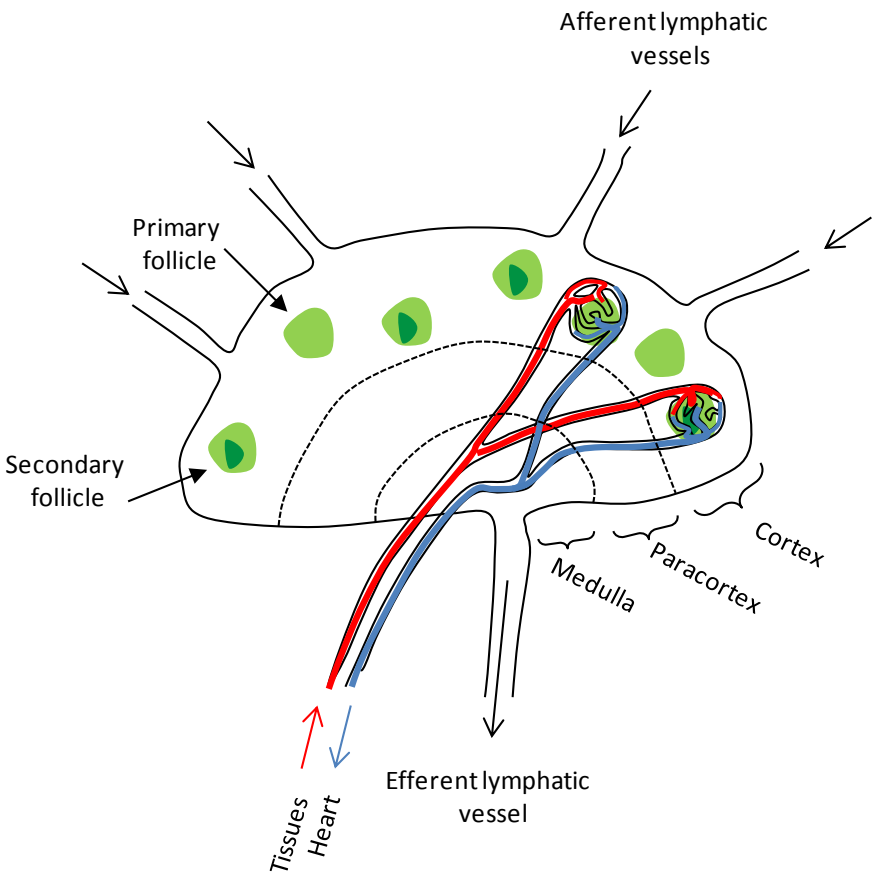

Figure 1.3 Lymph node organisation : the lymph node comprises three layers, the cortex, paracortex and medulla. Lymph flowing inwards via the afferent lymphatic vessels filters through the tiered structure and leaves via the efferent lymphatic vessel. Primary and secondary follicles (where memory T and B cells form) are found in the cortex.

1.3.4 T cell subsets

As mentioned above, $CD4^+$ T cells are typically depicted as being presented exogenous antigen on MHC Cl II molecules whilst $CD8^+$ T cells are presented antigen from MHC Cl I molecules. $CD8^+$ T cells are also termed cytotoxic T cells for their ability to initiate cell-mediated killing of pathogen-infected or tumour cells. For this reason, initiation of $CD8^+$ T cell immune responses during vaccination is especially important for vaccines targeting intracellular pathogens or cancer. $CD8^+$ T cells are also sentinel in antigen cross-presentation which allows exogenous antigen to stimulate $CD8^+$ T cell mediated immune responses (den Haan and Bevan, 2001). $CD4^+$ T cells can be further subdivided into numerous subsets initially chosen on the basis of their cytokine production (Mosmann et al., 1986). Today we recognise two traditionally distinct subsets of Th cells: Th1 and Th2 originally segregated based on their production of IFN-γ and IL-4 respectively. However there are also at least three further subsets of Th cells (Th17 (Dong, 2008), T_{FH} (Fazilleau et

al., 2009) and T_{Reg} (Piccirillo and Shevac, 2004)) which show considerable plasticity and have raised the question of whether T cells can be subdivided from a specific lineage (Bluestone et al., 2009). The variation in these numerous Th cell subsets further complicates our understanding of how TCR activation can lead to the T cell responses which are critical for immunity against pathogens.

It is known that immunity relies on the production of memory T cells which includes both $CD4^+$ and $CD8^+$ T cell subsets. These memory cells must be capable of rapidly becoming effector cells with the capacity to recognise previously encountered antigen and initiate its removal or breakdown (Kaech et al., 2002). More recent techniques have allowed cytokine profiling of Th and Tc cell subsets at different stages in their development to create a timeframe profile of cellular development (Seder et al., 2008). Using multiparameter flow cytometry, the model produced (Figure 1.4) shows how exposure of naïve T cells to antigen results in a progressive increase in cytokine production until a stage is reached whereby the cytokines produced are optimal for the cells function as an effector cell. During this process the cells are termed 'central memory' cells or T_{CM} cells and are positive for the cell surface homing receptor CCR7 indicating their ability to migrate to secondary lymphoid organs. Further exposure to antigenic stimuli results in a loss of CCR7 and cytokine production leading to decreased effector functions and ultimately apoptosis. The cytokines investigated for the purpose of developing this model were IFN-γ, TNF-α and IL-2 as they are all relevant cytokines in the development of immunity against many pathogens to which vaccine formulations are required (for example IFN-γ and TNF-α are both commonly used parameters to assess vaccine-related immune responses against pathogens such as *M. tuberculosis* and *Leishmania major*, whilst IL-2 has a role in T cell expansion). Multiparameter flow cytometry has been extensively implemented to assess T cell differentiation in studies involving development of vaccines against HIV (Kloverpris et al., 2009; Steers et al., 2009), urothelial carcinoma (Sharma et al., 2008), advanced stage melanoma (Perales et al., 2008) and TB (Duffy et al., 2009; Elvang et al., 2009; Kamath et al., 2009; Kamath et al., 2008a; Linderstrøm et al., 2009).

Figure 1.4 Schematic defining T cell function based on their ability to produce TNF-α, IL-2 and/or IFN-γ at periodic intervals during antigenic exposure. T cell progress from naïve $CD4^+$ T cells ($TNF\text{-}\alpha^-$ $IL\text{-}2^-$ $IFN\text{-}\gamma^-$) towards $IFN\text{-}\gamma^+$ T cells results in their progression from CCR7 expressing T_{CM} (central memory) cells towards T_{EM} (effector memory) cells which loose the cell surface homing receptor CCR7. Adapted from (Seder et al., 2008).

1.4 Vaccines

1.4.1 Types of vaccines

The Collins English dictionary defines a vaccine as 'a suspension of dead, attenuated, or otherwise modified microorganisms (viruses, bacteria, or rickettsiae) for inoculation to produce immunity to a disease by stimulating the production of antibodies'. This definition has somewhat changed recently with the development of cancer vaccines and vaccines against autoimmune diseases. Therefore, a more appropriate, simple and modern definition could be a substance which improves immunity to a certain disease. In their traditional organisation, vaccines are grouped into three categories: killed, attenuated or subunit vaccines. Killed vaccines contain chemically or heat-inactivated organisms which are unable to replicate or produce toxins. They structurally resemble their live counterparts however their genetic material is no longer functional. Typical examples of killed vaccines include inactivated polio vaccine (IPV) and the hepatitis A vaccine. Within the attenuated group are vaccines against yellow fever, mumps and TB. Attenuation is a method to weaken the pathogenic effects of a microorganism, either by altering its growth abilities or physically/chemically changing it so that it presents less of a danger to the host.

The final group comprises subunit vaccines which are composed of the antigenic unit of the pathogen. The principle benefits of subunit vaccines are their inability to revert to a pathogenic form, decreased toxicity, reproducible production and improved antigen specificity. Examples of subunit vaccines include vaccines against hepatitis B and influenza. In addition to this simplified list, vaccine development also includes toxoid based vaccines (against tetanus and diphtheria), peptide vaccines against autoimmune diseases (such as asthma and allergies), therapeutic cancer vaccines and DNA vaccines (whereby the host synthesises the antigenic proteins themselves).

As highlighted in Table 1.1, there is no clear rule as to which type of vaccine (recombinant/killed/attenuated etc) may be superior against a certain disease. Certainly the present aim of vaccinologists is to focus on safety, including low toxicity and prevention of reversion to virulence. However immunogenicity obviously plays a major role. With regards to smallpox, whilst the live attenuated composition of the vaccine led to the successful eradication of the disease, there were numerous other factors which certainly contributed including the lack of an animal reservoir, non-zoonotic disease, stability of the vaccine formulation and clear disease symptoms. However it is interesting to note that the side effects from this vaccine were also sufficiently damaging for the vaccination campaign in the US to be halted in 1972, 8 years prior to the declaration by the WHO that the world was free from smallpox (Kennedy et al., 2009). Therefore even the only vaccine to have ever led to the eradication of a disease had its faults and it is most probable that if the smallpox vaccine was still required today, it would not be used as it would fail clinical trials.

1.4.2 Improving subunit vaccine efficiency with adjuvants

1981 marked the appearance of the first marketed subunit vaccine against Hepatitis B (Hilleman, 2000). Therefore in terms of arrivals onto the vaccine market, the subunit vaccines are latecomers with development rather than discovery being hindered by their poor immunogenic profile. However they have the very important advantage over attenuated or killed vaccines in that there is no chance of reversion to a virulent form. Subunit vaccines are based on solely the antigenic epitopes originally derived from a virulent organism; there is a total loss of the molecules which would have typically alerted the host to the dangerous nature of the pathogen, in addition to the loss of particulate nature. This appears to be the downfall of subunit vaccines – they are simply too ‘clean’

and do not resemble pathogens. Therefore in an effort to improve the immune responses to subunit antigens, adjuvants are included in the formulation.

Adjuvants, whose name stems from the Latin 'adjuvare' meaning to aid, come in such a wide range of shapes and sizes that even though documentation on adjuvants has existed for nearly 100 years, there is still no universally approved grouping system. One of the first papers published by Ramon described the adjuvant effect of a range of compounds including tapioca, agar and starch oil (Ramon, 1925). Following this, the use of inorganic compounds including aluminium phosphate and aluminium hydroxide were documented (Glenny et al., 1926) and these aluminium based compounds became the first licensed adjuvants in commercial vaccines. Until late 2009, aluminium based compounds remained the only US-licensed adjuvants whilst in Europe a wider scope of adjuvants had been recognised (Table 1.2). However, adjuvant development from bench to marketed vaccines has been slow with the first non-aluminium salt adjuvant being licensed less than 15 years ago (Mbow et al., 2010; O'Hagan and Gregorio, 2009). The slow output and lack of successful licensing is due to numerous reasons including poor scale-up and inflexibility with regards to the scope of antigens with which they can be administered (O'Hagan and Gregorio, 2009). However when the varied immunising abilities of different vaccine adjuvants bearing remarkably similar structure and composition are considered, their slow development is hardly surprising. For these reasons, adjuvants are licensed in individual vaccines as opposed to being considered a separate entity. Some of the more common adjuvants are discussed in detail below.

Table 1.2 European licensed adjuvants for inclusion in vaccines

GSK, GlaxoSmithKline; MPL, monophosphoryl lipid A; HPV, human papillomavirus; HBV, hepatitis B virus; HAV, hepatitis A virus. Adapted from (Friede, 2009; Mbow et al., 2010).

1.4.2.1 'Alum' and other mineral salts

'Alum' is a collective term often used to refer to a group of aluminium salts including aluminium hydroxide, aluminium phosphate and aluminium potassium sulphate which are present in half of the vaccines currently in use in the US (National Center for Immunization and Respiratory Diseases. Centers for Disease Control and Prevention, 2009). The correct term for this group of adjuvants is aluminium salts; Alum actually refers solely to aluminium potassium sulphate (Marrack et al., 2009). The widespread use of aluminium salts as vaccine adjuvants is due to a combined ability to (generally speaking) improve vaccine immune responses as well as provide an excellent safety profile. Whilst occasional local reactions including inflammation, erythrema, subcutaneous nodules and allergic reactions are reported, when the numbers of people who have been vaccinated are considered, aluminium salts are exemplary adjuvants (Clements and Griffiths, 2002). The major downfall of aluminium salts is the polarised immune response which they activate being predominate activators of Th2 biased immunity. Aluminium salts are therefore ideal adjuvants in vaccines requiring strong humoral immunity with high levels of IgG1 antibodies and cytokines such as IL-4. Examples of this include vaccines against extracellular pathogens such as parasitic diseases (e.g. leishmaniasis) or toxoid-producing pathogens (e.g. diphtheria, tetanus). Among the methods used to mediate the Th1/Th2 balance, one has involved combining aluminium salts with adjuvants known as strong stimulators of Th1 responses. Examples include aluminium hydroxide combined with cationic liposomes (Agger et al., 2008a) or monophosphoryl lipid A (MPL), the latter of which is now a licensed adjuvant (AS04, GSK Biologicals).

It is only recently that the mechanisms by which aluminium salt adjuvants act have started to be uncovered, challenging the previous dogma that adjuvanticity was simply due to longer retention of antigen at the injection site, also known as the depot-effect (Marrack et al., 2009). The importance and mechanisms of antigen association to aluminium adjuvants remains debated (Iyer et al., 2003; Iyer et al., 2004; Morefield et al., 2005a; Morefield et al., 2005b) however it is clear that aluminium salts prevent the rapid removal and degradation of antigen normally seen upon injection of free antigen. There is a now a wide range of literature on the mechanisms of aluminium action (for in-depth reviews see (Brewer, 2006; Marrack et al., 2009)) which strongly suggest a role for the inflammasome and uric acid release upon local tissue damage (Kool et al., 2008; Marrack et al., 2009; O'Hagan and Gregorio, 2009). Briefly, injection of aluminium salts is known to cause tissue

damage and cell death with release of alarmins such as endogenous uric acid, infiltration of neutrophils and inflammatory mediators. Uric acid is capable of activating caspase 1, a component of the inflammasome, which can subsequently cleave pro-units of IL-1β and IL-18 to their active forms (Kool et al., 2008; Mariathasan, 2007; Martinon et al., 2002; Monie et al., 2009) (Figure 1.5). What is interesting is that aluminium salts are unable to activate monocytes and DCs *in vitro* (Kool et al., 2008; Sun et al., 2003), a property also seen by certain liposomal adjuvant formulations (Korsholm et al., 2006) (see Section 1.6 for an outline of liposomes).

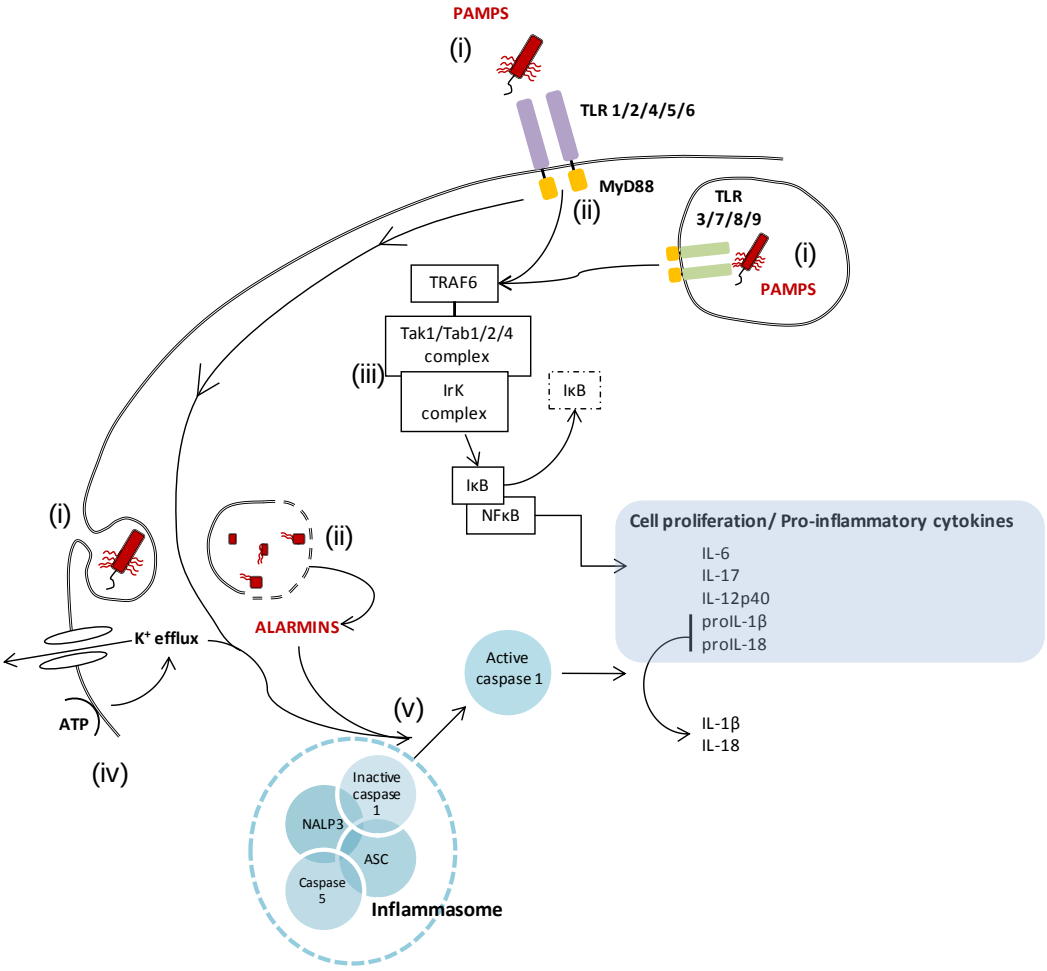

Figure 1.5 Mechanisms of immunological action of aluminium salts: downstream signalling events after activation of Toll like receptors (TLR) and the inflammasome. (i) Pathogens are endocytosed and activate TLRs via their pathogen associated molecular patterns (PAMPs) resulting in the initiation of intracellular events including release of alarmins and activation of MyD88 adapter proteins respectively (ii). The TRAF6 signalling pathway becomes activated leading to cleavage of IκB and activation of the transcription factor NFκB with production of pro-inflammatory cytokines such as IL-1β and IL-18, both of which are produced with 'pro' inhibitory domains (iii). Endogenous ATP can act as an alarmin to activate the $P2X_7$ receptor leading to potassium efflux from the cell (iv). The combination of potassium efflux and TLR activation lead to cleavage of the active caspase component of inflammasomes which is then able to cleave pro-domains of the cytokines IL-1β and IL-18 (v).

1.4.2.2 o/w emulsions: the success of MF59®

In 1997 the first emulsion adjuvant MF59® (Novartis, Italy) was licensed in Europe in the influenza vaccine Fluad®; to date more than 50 million people have been vaccinated against seasonal influenza with this vaccine (O'Hagan and Gregorio, 2009). MF59® is an oil in water (o/w) emulsion composed of 5 % squalene (naturally occurring oil) combined with surfactants sorbitan trioleate (Span 85) and polyoxyethylenesorbitan monooleate (Tween 80). Despite concerns regarding its safety due to the occurrence of autoimmune dysfunctions in rats (Carlson et al., 2000), the adjuvant has a established safety profile in humans of good-health and immunocompromised populations (Black et al., 2010; Donatoa et al., 1999). It is interesting to note that MF59® is not the first emulsion to be licensed for human use; the well-known experimental adjuvant Incomplete Freund's adjuvant (ICF) (Freund et al., 1948) was once used in human influenza vaccines (Chang et al., 1998). In contrast to MF59®, ICF is a water in oil (w/o) emulsion composed of a light mineral oil (such as Bayol F) and the emulsifier mannide monooleate (Aracel A™), combined in a 9:1 volume ratio (Lindblad, 2000). Whilst it has been withdrawn from human use due to occasional serious local reactions, it remains an experimental "gold-standard" adjuvant in immunisation protocols. MF59® has now been in use for over 10 years with its success being attributed to the rendering of soluble antigen to particulate form, improved cell recruitment to the injection site and antigen uptake with transport to local lymph nodes (Mosca et al., 2008). Similar to aluminium salts, no direct activation of DCs has been noted although improved trafficking and antigen uptake by macrophages and DCs respectively has been noted, and expression of soluble activation factors may indeed lead to indirect DC activation (Dupuis et al., 2001; Mosca et al., 2008). Currently MF59® is only used in influenza vaccination, however, a recent report combining MF59® with enveloped glycoproteins from hepatitis C virus (HCV) has shown success in human Phase I clinical trials (Frey et al., 2010).

1.4.2.3 Taking tips from pathogens with MPL

Monophosphoryl lipid A (MPL) is the first licensed adjuvant containing bacterial analogues which are known to act via TLRs. Derived from lipopolysaccharide (LPS) covered Gram negative bacterial cell surfaces, MPL is a synthetic and slightly modified version of the anchor moiety of LPS, 'lipid A' (Alving and Rao, 2008). MPL has been licensed by GSK Biologicals for use in its 'Adjuvant Systems' including AS01, AS02 and AS04, all of which

contain MPL (Casella and Mitchell, 2008). In terms of development, the most successful Adjuvant System is AS04 which was successfully licensed in the US in October 2009 in the vaccine Ceravix™ targeting human papillomavirus (HPV) types 16 and 18. AS04 is also included as an adjuvant in recombinant hepatitis B (HBV) vaccine Fendrix™ (although this is not licensed in the US yet) and is in Phase III clinical trials in a herpes simplex 2 virus (HSV) vaccine (Didierlaurent et al., 2009). It is important to note however that in all of the above mentioned adjuvant systems, MPL is combined with a secondary adjuvant, namely liposomes and QS21 (AS01), QS21 alone (AS02) or aluminium hydroxide (AS04). The inclusion of these secondary adjuvants is due to the requirement of MPL and especially the antigen to have a delivery system which MPL alone cannot contribute.

MPL, and its natural analogues lipid A and LPS, act via TLR4 present on the cell surface with resulting activation of a series of intracellular pathways including MyD88 and NFκB, finally resulting in transcription of co-stimulatory molecules, chemokines and cytokines which express a predominantly Th1 phenotype (Didierlaurent et al., 2009). The finding that MPL could be used and indeed licensed as an adjuvant has been very important for the development of novel adjuvants as it has led to the active pursuit of molecules that are ligands for TLRs and other cellular receptors. By purposefully activating such receptors the adjuvant is mimicking pathogenic actions and therefore if the ligand:receptor combination is carefully selected, a similar immune response that would normally be initiated against the pathogen of interest can be mounted. The different TLRs and their intracellular signalling pathways will be discussed in further detail in Section 1.5, however, on a very general note it can be said that their activation leads to initiation of important innate immune responses that can mature APCs and consequently drive acquired immunity.

1.4.2.4 Antigen delivery with vesicular systems

There are numerous lipid and polymer based delivery systems which exhibit a spherical or vesicular form, thereby allowing antigens to be entrapped or surface-associated depending on the physicochemical properties of each component. Examples of such include microspheres, nanoparticles, virosomes, immune-stimulating complexes (ISCOMS), liposomes and bilosomes among others. By associating antigens with such delivery systems, the antigen can be protected from the extracellular milieu thereby limiting

peptide/protein and nucleic antigen breakdown by enzymes, as well as preventing the rapid removal of such small compounds by the mononuclear phagocytic system (MPS).

Nucleic antigens are especially prone to enzymatic degradation by nucleases and the poor transfection noted upon application of free DNA to cells led Felgner and colleagues to develop Lipofectin™ as a transfection agent (Felgner et al., 1987). Whilst Lipofectin™ makes use of liposomes (discussed in detail in section 1.6) as a method to condense, associate with and deliver DNA intracellularly, other particulate systems exist which conduct a similar role as antigen/drug delivery vehicles. Nanoparticles (named as such due to their sub-micron size) and microspheres are both spherical particles used extensively as drug delivery systems as they reduce drug-associated toxicity, improve drug solubility and can improve bioavailability (Perrie and Rades, 2010). In addition, both nanoparticles and microspheres can be used for vaccine antigen delivery (Kirby et al., 2008b; Mohanan et al., 2010) although the small solid nature of nanoparticles restricts antigen association to the surface of the vesicles. Nanoparticles and microspheres are commonly composed of biodegradable polymers such as poly(D,L-lactide-co-glycolide) (PLGA) or polylactic acid (PLA) which can also be combined with poly(ethyleneglycol) (PEG) to form 'stealth' vesicles capable of avoiding removal by the MPS. Other particulate delivery systems capable of associating with drugs and vaccine antigens include dendrimers which are composed of a polymeric spherical lattice (Dutta et al., 2010) and ISCOMS composed of phospholipid, cholesterol and saponins which form cage-like structures (Myschik et al., 2006).

1.5 Improving our Understanding of How Adjuvants Work

1.5.1 Diverse substances with a diverse classification

Generally speaking, adjuvants can be divided into groups relating to their physical properties, such as inorganic salts, liposomes, oil in water (o/w) emulsions, surfactants etc. This classification poses the problem that the method in which the adjuvant acts is unknown and therefore does not help with eliminating or identifying future adjuvant candidates. Another classification which will become more complex with time is proposed by O'Hagen and De Gregorio (O'Hagan and Gregorio, 2009) whereby adjuvants are classed as 1st or 2nd generation adjuvants. In this system a 1st generation adjuvant refers to one of the more traditional substances normally composed of one immunostimulatory compound. These include aluminium salts, liposomes and MF59. Addition of further

immunostimulatory compounds to these existing 1st generation adjuvants results in a 2nd generation adjuvant such as the aforementioned adjuvant systems (GSK Biologicals), IC-31® (Intercell, Austria) and ISCOMS. Whilst the possibility exists to extend the system to 3rd generation adjuvants (and 4th and 5th etc), the system becomes increasingly awkward and does not give any indication as to how the adjuvants may work. In 2000 Virgil Schijns proposed a system whereby adjuvants can be divided into groups depending on their method of immunostimulatory action (Schijns, 2000). Whilst five groups were suggested, it is possible that further mechanisms may be deduced in the future, in addition to the difficulty in classification when one adjuvant has more than one mode of action. Finally and possibly the simplest involves a classification system based on whether the adjuvant works via TLRs or not (Mosca et al., 2008). In this instance only two groups exist (TLR-dependent and TLR-independent) however for adjuvants containing two or more immunogenic components (such as GSK's AS04 adjuvant containing aluminium hydroxide and MPL), they may act via both TLR-dependent and TLR-independent mechanisms further complicating matters. For the purposes of identifying the mechanisms of action of present and future adjuvants, the classification system by Schijns is the most appropriate and the basis of how these adjuvants will be described herein.

1.5.2 Concepts of adjuvant mediated immunogenicity

According to Schijns classification, there are five methods in which adjuvants may be immunostimulatory. Highlighted in Figure 1.6, these methods provide an ideal way to explain the immune system with relevance to systemic delivery of vaccines.

Upon parental vaccine delivery there is localised tissue damage which results from the physical insertion of the needle and vaccine components into the tissue milieu. Cells will inevitably be ruptured releasing their intracellular contents including mitochondria, uric acid and heat shock proteins (HSPs), all of which are termed 'alarmins' (Bianchi, 2007). Alarmins are host-derived substances released upon non-intentional cell death (in contrast to apoptosis) and, unsurprisingly signal to the host that there is a problem such as tissue destruction or stress. This can be considered the first mechanism of adjuvant action (Shi et al., 2000) and is termed the danger signal, in reference to the 'danger model' originally described by Matzinger (Matzinger, 1994). The danger model assumes that the host can differentiate between harmless and harmful as opposed to self and non-self (the later model originally described by Metchnikoff and thoroughly reviewed by Janeway (Janeway,

1992)). Overlapping with the danger model is the concept of signal 0, the second class by which adjuvants act. Signal 0 can include alarmins but with respect to adjuvant composition and formulation, the signal 0 group principally includes exogenous pathogen associated molecular patterns (PAMPs). Importantly the signal 0 concept is the physical binding of alarmins or PAMPS (collectively termed danger associated molecular patterns, or DAMPS (Bianchi, 2007)) to pathogen recognition receptors (PRRs) and the resulting intracellular signalling cascade leading to activation of the cell (see Figure 1.7 for a schematic of DAMPS and their TLRs). PRRs are constitutively expressed on or within cells of the innate immune system (Schijns, 2000) and include, among others, TLRs, inflammasomes, integrins, c-type lectins and antibody Fc receptors. The specific signalling cascades initiated by these DAMP-PRR interactions will be discussed later but some of the resulting actions include the production of pro-inflammatory cytokines, chemokines and co-stimulatory molecules required for the productive activation of the T cell upon antigen presentation. In fact, antigen presentation without simultaneous co-stimulatory molecule interactions has been shown to lead to tolerance. Co-stimulatory molecules such as CD80 and CD86 are consequently very important for successful T cell activation and whilst being expressed constitutively on DCs, they are only expressed on other APCs including macrophages and B cells upon activation. Adjuvants which are able to activate macrophages and B cells to induce expression of CD80 or CD86, and those able to upregulate expression of the said markers on DCs can be grouped under the third adjuvantal category, 'recombinant signal 2'. The principle adjuvants in this group are cytokines which can be considered as endogenous adjuvants and have also been administered with vaccines experimentally (see (Boyaka and McGhee, 2001; Egan and Israel, 2002; Heath and Playfair, 1992) for review articles), as well as TLR-signalling adjuvants.

The final two adjuvantal categories relate more to the physical structure of the adjuvant and its physical localisation *in vivo*. As discussed previously, there is much diversity in the structures of adjuvants although many of them are based on emulsions or vesicular structures. The benefits of vesicular structures are two-fold; firstly they can be used to 'carry' antigen, either by entrapment or adsorption; secondly vesicles are naturally occurring and appropriately sized structures that can be endocytosed by cells depending on their composition and size (see section 1.3.2 and Figure 1.1), making them ideal intracellular delivery systems. This paradigm of assisting antigen uptake is the fourth

adjuvantal category and principally concerns liposomes, nanoparticles, microspheres, virosomes, emulsions and ISCOMs, all of which are able to package antigen into a delivery vehicle. The final concept is the idea that antigen held at the injection site for a long period of time results in lengthy presentation of antigen to innate immune cells. Known as the depot-effect, it refers to localisation of antigen (with or without adjuvant) at the injection site and not in the lymphoid organs (although increased presentation of antigen in the lymphoid organs may be a direct consequence of the depot effect and is often the desired effect). The depot-effect is therefore dependent on numerous factors such as the route of injection, the tissue found at the injection site and characteristics associated with the formulation itself such as viscosity and particulate size.

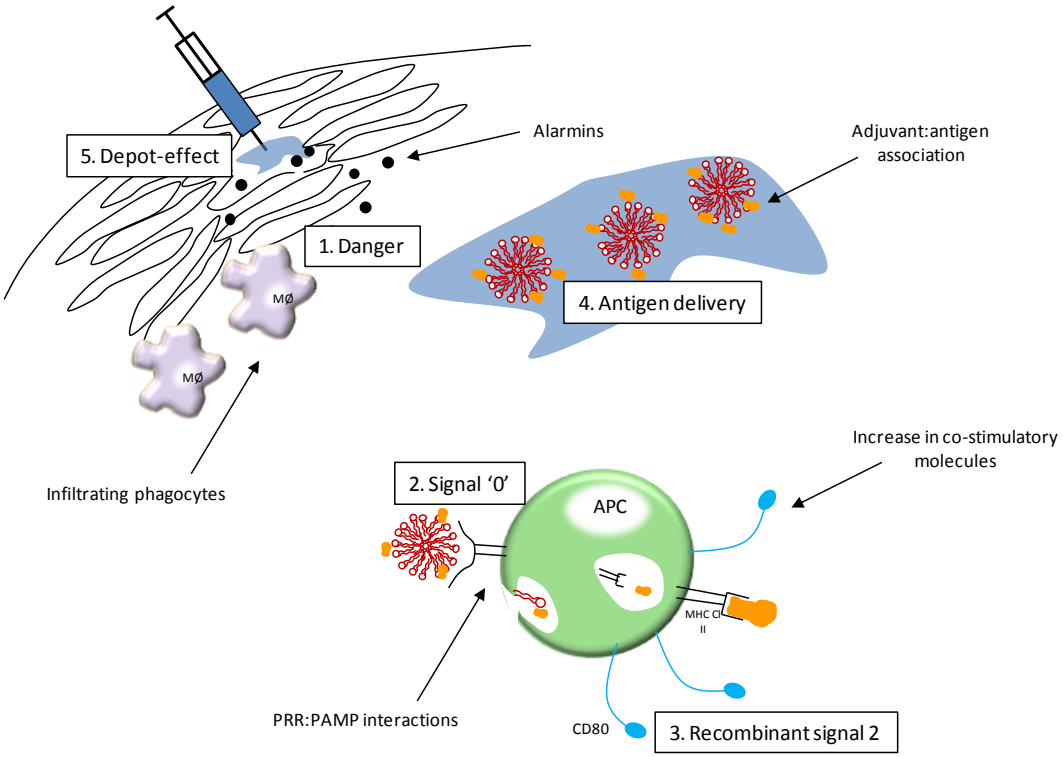

Figure 1.6 Classification of adjuvants: the five steps of adjuvant action are described in the text. APC, antigen presenting cells; MØ, macrophage; PRR, pathogen recognition receptors; PAMP, pathogen associated molecular patterns.

Figure 1.7 Flow chart showing pathogen associated molecular patterns (PAMP) and alarmins which are known to bind Toll like receptors (TLRs). Synthetic analogues known to target specific TLRs are **bold underlined**. R-837, Imiquimod; R-838, resiquimod; HSP, heat shock protein; HMGB1, high motility group box 1; ECM, extracellular matrix. Data obtained from a range of sources including (Bianchi, 2007; Dockrell and Kinghorn, 2001; Monie et al., 2009; O'Hagan and Gregorio, 2009)

1.5.3 Defining the ideal adjuvant

From this analysis of different 'types' of adjuvants, it is clear that there are still many overlaps between groupings however the system devised by Schijns does help to define the mechanisms by which adjuvants work. With this in mind, it is possible to envisage how the perfect adjuvant would work. Firstly, it must contain a sufficient amount of PAMPs to alert the immune system of danger but without causing hyper-immunostimulation which may result in anaphylactic shock or local tissue damage by excess of inflammatory mediators. Too few PAMPs or PAMPs without sufficient toxicity may not stimulate sufficient production of pro-inflammatory molecules or chemokines which are vital to alert circulating APCs. It may just be that a certain degree of local tissue damage is also beneficial as it allows the release of alarmins, further promoting APC influx to the injection site (Shi et al., 2000). The adjuvant should also ideally stay associated with the antigen until uptaken by APCs so that the immune system can make a collective association of both components. Free antigen, in the form of nucleic material or small peptide antigens, is rapidly degraded by extracellular enzymes and many subunit protein vaccine antigens are themselves immunogenically inert. Protein and peptide antigens undergo rapid

removal via the circulatory or lymphatic system and without any danger signals attached, the protein may simply be removed before antigen uptake and presentation can occur. Whilst it appears important for adjuvant and antigen to stay associated until arrival of APCs, whether antigen uptake should occur simultaneously with the adjuvant is unknown and is discussed in more detail in Chapter 5.

1.6 Liposomes: The Ideal Adjuvant?

1.6.1 Liposomes as delivery systems

Originally discovered in 1965 by the late Alex Bangham, liposomes are essentially bilayered vesicles composed of lipids, with or without additional lipophilic components, which form upon exposure to aqueous media (Bangham et al., 1965). Since their discovery, liposomes have been recognised as efficient drug and vaccine antigen delivery systems due to their ability to encapsulate or surface adsorb a wide variety of molecules. Much of the pioneering work on the subject of liposomes was undertaken by Gregoriadis who highlighted the ability of liposomes to entrap drugs (Gregoriadis, 1973), described methods to improve liposomal stability (Gregoriadis and Davis, 1979) and discussed the problems associated with liposomal clearance *in vivo* (Gregoriadis, 1995a, b; Gregoriadis and Senior, 1980). Furthermore, many of the positive aspects of liposomes as drug delivery vehicles can also be applied to vaccine antigen delivery. Firstly liposomes can protect antigen from extracellular degradation via either entrapment or surface adsorption (Gregoriadis et al., 1999). Secondly liposomes can be formulated to have an anionic, neutral or cationic surface charge which will determine what type of antigens may associate, in addition to how the liposome will interact with soluble and cellular components of the host (discussed in detail in Chapter 4). Thirdly liposomes are incredibly flexible systems as their ampiphilic structure allows for incorporation of both hydrophilic and lipophilic molecules. For example, whilst anionic nucleic acids and peptides/proteins can be surface adsorbed to cationic vesicles, soluble antigens can be encapsulated in uni- or multilamellar vesicles and lipophilic glycolipids and bacterial cell wall derivatives are able to insert into the bilayered membrane (Figure 1.8).

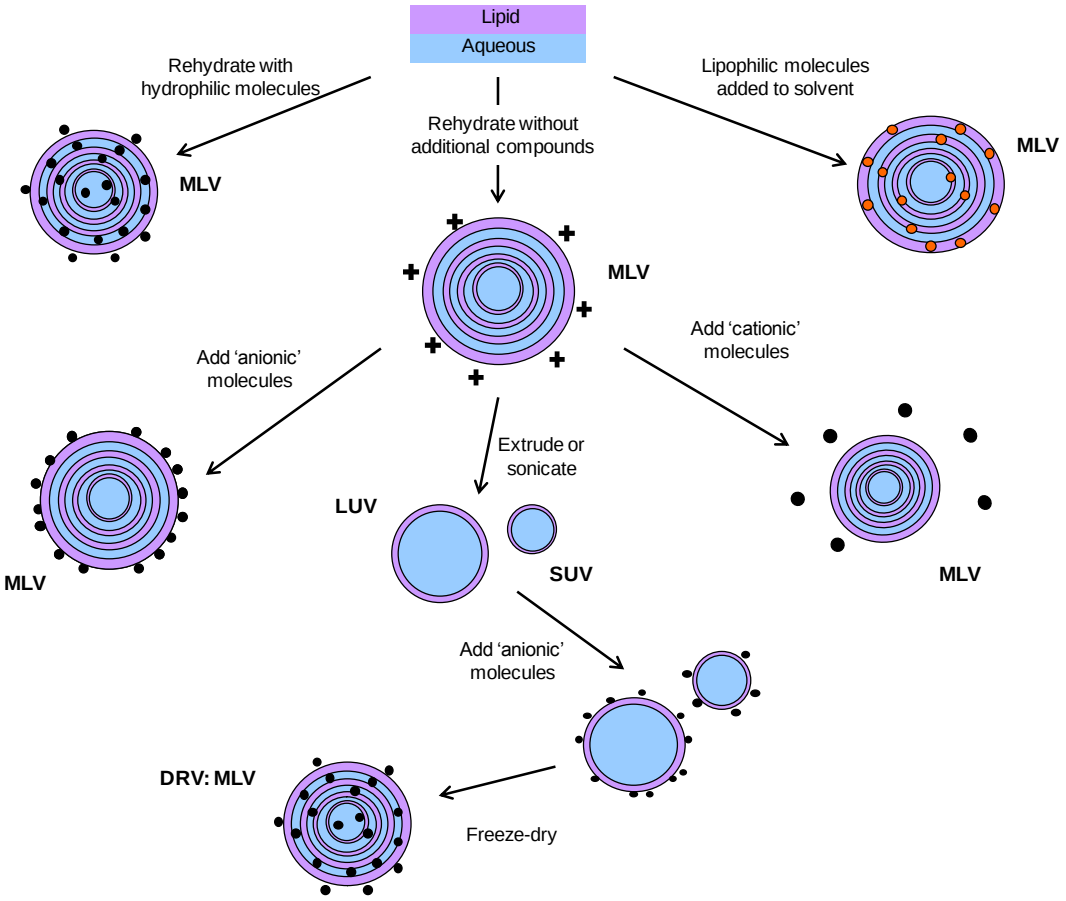

Figure 1.8 Schematic of liposome types including multilamellar vesicles (MLVs), small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs). A further type of liposome can be described as a dehydration-rehydration vesicle (DRV) which forms after freeze-drying and is used to encapsulate antigen.

1.6.2 Cationic liposomes expressing immunostimulatory properties

In addition to being successful drug and antigen delivery systems, liposomes expressing a positive charge have also been shown to have specific immunostimulatory abilities, independent from their delivery vehicle properties (Figure 1.9 shows a selection of cationic lipids commonly used in liposome formation). The immunostimulatory properties of cationic lipids were originally documented in a screening study by Gall (Gall, 1966). Within this study a wide range of compounds were investigated for their ability to adjuvant diphtheria or tetanus toxoids in guinea pigs. Compounds included non-ionic, anionic and cationic surface acting agents, amines, guanidines, benzamidines, thioureas, thiosemicarbazides, thiosemicarbazones, thiouroniums and various nitrogenous bases. With regard to the group of surface acting agents, or more commonly termed surfactants, Gall observed increased adjuvanticity for those expressing cationic quaternary ammonium

head groups and long alkyl chains (Gall, 1966). These lipids are able to form bilayered vesicles upon exposure to aqueous environments, a process which is attributed to the geometry of the lipid known as the critical packing parameter (CPP). The CPP defines the final lipid-structure that will form and is dependent on the length and saturation of the lipid hydrocarbon chains, as well as the size of the lipid head group (Perrie and Rades, 2010). The majority of immunostimulatory cationic lipids form liposomes alone, or with the inclusion of a neutral helper lipid primarily included to stabilise the membrane (discussed in further detail in Chapter 3). Such cationic liposomes are of principle importance in gene delivery and experimental vaccines involving both nucleic acid antigen (termed lipoplexes when associated with the surface of liposomes) and peptide/protein antigens.

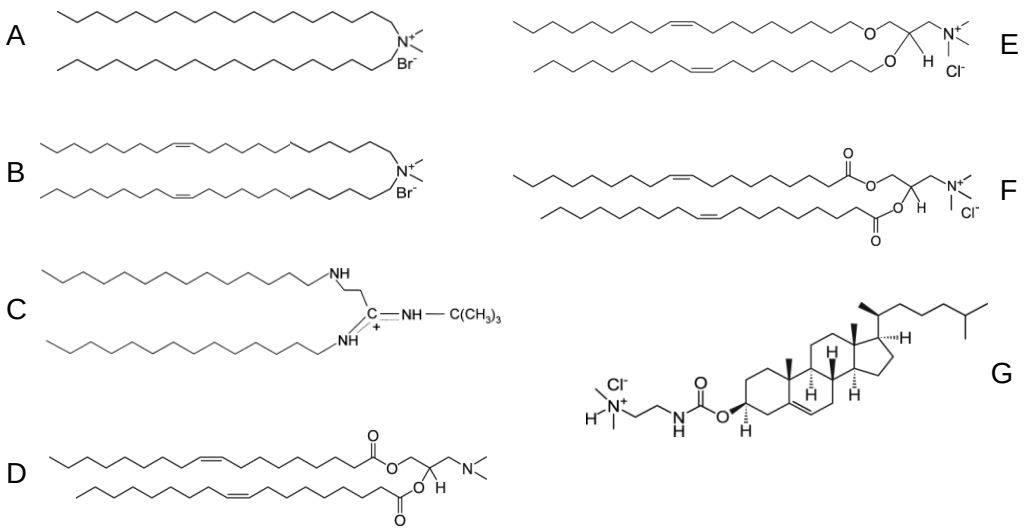

Figure 1.9 Commonly used cationic lipids expressing transfection or immunostimulatory abilities. A, dimethyldioactadecylammonium (DDA); B, N,N-dioleyl-N,N dimethylammonium (DODA); C, DiC14-amidine; D, 1,2-dioleoyl-3-dimethylammonium-propane (DODAP); E, 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA); F, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP); G, 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol hydrochloride (DC-Chol). Diagrams were obtained from www.avantilipids.com.

1.6.3 Liposomal adjuvants

Although there are no liposomal adjuvanted vaccines currently on the market, their use in laboratory transfection (e.g. Lipofectamine™) is commonplace (see (McNeil and Perrie, 2006) for a review on liposomal gene delivery) and there also exists numerous liposomal

formulations designed as delivery systems for chemotherapeutic and antimicrobial agents (Gregoriadis, 1973, 1985, 1995a; Perrie, 2005).

The lack of liposomal adjuvants on the market is not without trying however and again there is a wealth of research dating from the 1980's and 1990's based on improving immune responses with the aid of liposomes (Bakouche and Gerlier, 1986; dal Monte and Jr, 1989; Gregoriadis, 1985, 1994; Gregoriadis et al., 1996; Nakanishi et al., 1997; Oussoren et al., 1997). It was not however until the mid- 1990's that the importance of liposomal cationic charge was noted for the induction of strong immune responses. Since then research on cationic liposomal adjuvants has also diversified towards cationic structures such as ISCOMS/PLUSCOMS (Lendemans et al., 2007; McBurney et al., 2008) and the cationic peptide $KLKL_5KLK$, a component of IC31® adjuvant (Schellack et al., 2006). IC31® is currently in Phase I and Phase II clinical trials (Table 1.1) due to its success with the tuberculosis antigens Ag85B-TB10.4 and Ag85B-ESAT-6 respectively (Agger et al., 2006; Kamath et al., 2008a; Kamath et al., 2008b).

Returning to cationic liposomes, much focus has been in trying to elucidate the mechanisms by which these vesicles confer significantly enhanced immune responses compared with controls such as aluminium salts or ICF. Whilst the cationic lipids 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 3β-[N-(N',N'-dimethylaminoethane)-carbomyl] cholesterol (DC-Chol) will be focussed on in greater detail in Chapter 5, a brief point to make with regards to their mechanisms of action would be that nothing is as simple as it would seem! Whilst DOTAP, DC-Chol and many other cationic lipids share numerous physicochemical properties, there is great variation in their immunogenic abilities and methods by which they target the immune system. The focus here, and indeed throughout this thesis, is on dimethyldioctadecylammonium bromide (DDA) which was initially noted for its immunogenic abilities in the aforementioned study by Gall (Gall, 1966). DDA is a rather simple quaternary ammonium lipid possessing two saturated 18-carbon lipophilic tails which promote bilayer formation in aqueous solution. The head group is also relatively small when compared to DOTAP, DC-Chol and other cationic lipids with documented immunostimulatory action (Figure 1.9). DDA liposomes have been tested in combination with a wide range of additional molecules used either to stabilise the vesicles or to increase the immunogenic responses towards the vaccine antigen; this forms the basis and is discussed in detail in Chapter 3. With respect to

liposomes composed solely of DDA, improved immunogenicity in response to vaccine antigen has been noted in numerous disease models including TB (Andersen, 1994; Davidsen et al., 2005; Holten-Andersen et al., 2004; Lindblad et al., 1997; Rosenkrands et al., 2005), malaria (Desowitz and Barnwell, 1980), tetanus (Stanfield et al., 1973) and listeriosis (Van der Meer et al., 1979). The adjuvant action noted by DDA liposomes is understood to be due to a combination of the following: a strong antigen depot-effect, rendering antigen to a particulate form, improving antigen delivery to secondary lymphatic organs and promoting antigen uptake by APCs (Hilgers and Snippe, 1992; Korsholm et al., 2006). Whilst a specific cellular receptor for DDA liposomes capable of initiating an intracellular activation pathways has yet to be discovered, it is comprehendible that cell surface receptors play an important role in liposome uptake via endocytotic mechanisms.

1.7 Aims of Work

As discussed above, there is a considerable lack of effective vaccines to target the most significant causes of infectious disease. Furthermore, despite the many decades of research into the mechanism of adjuvantal action, we still do not have a clear and complete understanding into how these substances can make the host mount a sufficiently strong immune response against a small peptide/protein/nucleic acid to consequently protect from infectious disease. By gaining a better understanding into the mechanisms by which adjuvants act it is hoped that improved adjuvant and vaccine design with a more structured approach may lead to more productive formulations going into clinical trials.

One of the lead TB adjuvants is a cationic liposome formulation ‘CAF01’ composed of DDA and trehalose 6,6’-dibehenate (TDB) (Table 1.1). Little is known however about the way in which this adjuvant is effective. Focusing on this formulation and other cationic liposomes sharing properties with CAF01, the aim of this work was to investigate further how CAF01 acts as an adjuvant. To achieve this aim the overall objectives of the work were:

- Screen a range of cationic liposome formulations to investigate the immunogenicity of these cationic liposomes thereby allowing further optimisation of the systems.

- Measure the key pharmaceutical properties including physicochemical characteristics and stability.
- Investigate the biodistribution of liposomes and their associated antigens with the aim to correlate these to their physicochemical properties and immunological function.
- Use of cell lines to investigate the immunostimulatory effects of various cationic liposomes *in vitro* and correlate these with *in vivo* responses.

Chapter 2: Materials and Methods

2.1 Materials

AbD Serotec, Oxford, UK: Goat anti-mouse IgG1:HRP (STAR132P), goat anti-mouse IgG2a:HRP (STAR133P), goat anti-mouse IgG2b:HRP (STAR134P), goat anti-mouse IgG:HRP (STAR77), murine monoclonal isotyping test strips (MMT1)

American type culture collection (ATCC): J774.1 and RAW264.7γNO(-) monocyte/macrophage cell lines

Avanti Polar Lipids, Alabaster, US: Dimethyldioctadecylammonium bromide (DDA), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 3β-[N-(N',N'-dimethylaminoethane)-carbomyl] cholesterol (DC-Chol), distearoyl-glycero-phosphatidylcholine (DSPC), trehalose 6,6'-dibehenate (TDB), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rho-DPPE)

Beckman Coulter, High Wycombe, UK: OptiLyse®C Lysing solution

BioRad, Hemel Hempstead, UK: Silver stain kit

Biosera, Leicestershire, UK: Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, foetal bovine serum (FBS), penicillin-streptomycin-glutamine (PSG)

Enzo Life Sciences, Exeter, UK: Vitamin D3 (VD3), Phorbol 12-myristate 13-acetate (PMA)

Fisher Scientific, Leicestershire, UK: Methanol and chloroform (both HPLC grade)

GE Healthcare, Amersham, UK: 1,2-dipalmitoyl L-3-phosphatidyl[N-methyl-^{3}H]choline (^{3}H-DPPC), [methyl-^{3}H]Thymidine

Geneflow, Staffordshire, UK: EZ-PCR Mycoplasma test kit

Greiner, Stonehouse, UK: Plasticwear for cell culture experiments

IDN Biomedical Inc, Aurora, Ohio, US: Tris-base

Invitrogen, Paisley, UK: Tris-glycine gels (12 %), broad range molecular marker

Medicell International Ltd, UK: Dialysis membrane (12-14 kDa pore size)

Merck Chemicals Ltd, Nottingham, UK: Ovalbumin, 5X crystalline

Perkin Elmer, Waltham, MA, US: ^{125}I (NaI in NaOH solution), Solvable™, Ultima Gold scintillation fluid

Pierce Biotechnology, Rockford, IL: Pierce iodination tubes containing Pierce iodination reagent (formally known as IODO-GEN®)

Promega, Southhampton, UK: CellTiter 96 AQueous non-radioactive cell proliferation assay (MTS assay), CytoTox 96 non-radioactive cytotoxicity assay (LDH assay)

R+D, Abingdon, UK: Mouse DuoSet capture ELISA IL-2 (DY402), IL-5 (DY405), IL-6 (DY406), IL-10 (DY417), IFN-γ (Dy485)

Sigma-Aldrich, Poole, UK: Cholesterol (Chol), PBS tablets, ovalbumin (OVA), bovine serum albumin (BSA), lysozyme (from hen egg white), bicinchoninic acid (BCA) protein assay kit, Triton® X-100, hydrogen peroxide, Sephadex™ G-75, 2'2 azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid, concanavalin A (ConA), β-N-Acetylglucosaminidase Assay kit (NAG assay), fluorescein isothiocyanate (FITC), bromophenol blue, β-mecaptoethanol, glycerol, sodium dodecyl sulphate (SDS), glycine, brilliant blue R, pontamine blue, neutral red, sodium azide, citric acid, dimethyl sulphoxide (DMSO), tryphan blue, ethylenediaminetetra-acetic acid (EDTA), heparin, Incomplete Freund's Adjuvant (IFA), potassium chloride, potassium phosphate, sodium chloride, sodium phosphate dibasic, sulphuric acid, tetramethylbenzidine, Trizma base, Tween-20

Statens Serum Institute, Copenhagen, Denmark: Non-His-tagged protein Ag85B-ESAT-6 was produced as described by Olsen et al., (Olsen et al., 2001) , CTH1 was kindly donated by Frank Follmann, N,N-dioleyl-N,N dimethylammonium Chloride (DODA)

VWR International, Leicestershire, UK: Terumo Insulin needles

Ethics Approvals for *in vivo* studies

All experimentation undertaken at Aston University strictly adhered to the 1986 Scientific Procedures Act (UK). All protocols have been subject to ethical review and were carried out in a designated establishment. Mice were obtained from Charles River.

All experimentation undertaken at Statens Serum Institute, Denmark strictly adhered to the regulations of the Danish Ministry of Justice and animal protection committees and was in compliance with European Community Directive 86/609. All protocols have been subject to ethical review and were carried out in a designated establishment. Mice were obtained from Harlan Scandinavia, Allerod, Denmark.

2.2 Methods: Preparation of Liposomes

2.2.1 The lipid-film hydration method

Liposomes were produced using the lipid-film hydration method originally described by Bangham (Bangham et al., 1965). This relatively simple method was chosen based on previous work by Davidsen et al. in which the vesicle size of liposomes produced by the lipid-film hydration and aqueous heat methods was investigated (Davidsen et al., 2005). Addition of the stabilizing and immunostimulatory component trehalose 6,6'-dibehenate

(TDB), in addition to antigen, had no effect on the size of vesicles produced by the lipid-film hydration method. In contrast, those produced by the aqueous heat method showed significant increases in size upon addition of either component (Davidsen et al., 2005).

To produce liposomes, lipids at appropriate ratios were first dissolved in solvent (chloroform:methanol 9:1) in a round-bottom flask (rbf); throughout these studies lipids stocks were made to 10 mg/ml whereas TDB was made to 2 mg/ml. The organic solvent was then evaporated on a rotary evaporator (Buchi rotavapor-R) to obtain a dry film. Residual solvent was removed using a stream of N_2 after which the lipid-film was hydrated using buffer at a temperature dependent on the lipid transition temperature and at least 10°C above it. Table 2.1 presents the transition temperatures of the lipids used. For most work Tris buffer (10 mM, pH 7.4) was used to rehydrate lipid films however occasionally PBS buffer was used and is marked as appropriate. Rbfs were sealed with parafilm to prevent buffer evaporation. The lipids were hydrated for approximately 20 mins with frequent vortexing to encourage lipid mixing and vesicle formation.

When TDB was incorporated into formulations, the ratio of lipid:TDB used was 5:1 (weight) or 8:1 (molarity). In characterisation studies the lipid concentration was 1.25 mg/ml however for various other applications (indicated as appropriate) the concentration of liposomes was increased to as much as 10 mg/ml.

To reduce the size of liposomes produced by the lipid-hydration method, liposomes were sonicated at 4 microns for between 30-120 seconds, depending on the volume of liposomes used. For studies in which antigen was added to the liposomes, sonication took place prior to addition of antigen.

A novel method was used to produce ‘big’ liposomes; a high amount of lipid (10-fold more than normal) was placed in a very small rbf (5 ml volume) and a lipid film produced using the lipid-film hydration method. The lipid film was hydrated in a volume of buffer 10-fold less than usual and vortexed. Preliminary studies showed that the combination of small rbf, high amount of lipid and low rehydrating buffer were all required for the successful formation of large liposomes.

Table 2.1 Transition temperatures of lipids used for the formation of liposomes

Lipid	Transition temperature (°C)
Dimethyldioctadecylammonium bromide (**DDA**)	+ 47 (Davidsen et al., 2005)
N,N-dioleyl-N,N dimethylammonium chloride (**DODA**)	n.a
1,2-dioleoyl-3-trimethylammonium-propane (**DOTAP**)	- 12 (Zuidam et al., 1999)
3β-[N-(N',N'-dimethylaminoethane)-carbomyl] cholesterol (**DC-Chol**)	n.a
Distearoyl-glycero-phosphatidylcholine (**DSPC**)	+ 55 (www.avantilipids.com)

n.a; not available.

2.2.2 Production of radiolabelled liposomes

In order to track the movement of liposomes *in vivo*, liposomes were radiolabelled by incorporating tritiated lipid 1,2-dipalmitoyl L-3-phosphatidyl[N-methyl-^{3}H]choline (^{3}H-DPPC) which is readily incorporated into the bilayer of liposomes (Henriksen-Lacey et al., 2010a). Liposomes including ^{3}H-DPPC were produced using the lipid-film hydration method described in section 2.2.1 with the addition of ^{3}H-DPPC (supplied in solvent) to the lipid mixture prior to solvent removal. The amount of ^{3}H-DPPC added was based on the radioactivity (^{3}H) and the concentration of DPPC in the liposomes so that the physicochemical liposomal properties were not altered. The specific activity of ^{3}H-DPPC was 2.85 TBq/mmol with a radioactive concentration or 37 MBq/ml. This high ratio of ^{3}H:DPPC was ideal for biodistribution work as both factors - high radioactivity and low DPPC - were fulfilled. ^{3}H-DPPC was incorporated into liposomes at ^{3}H-DPPC:lipid weight ratio of 1:10,000. This is equivalent to 25 ng DPPC/dose with an approximate radioactivity of between 100-200 kBq.

2.2.3 Production of fluorescently labelled liposomes

Fluorescently labelled liposomes were used in flow cytometric and fluorescence microscopy studies. The lipid 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rho-DPPE) was incorporated into liposome bilayers using the same lipid-hydration method as discussed in section 2.2.1. Rho-DPPE was dissolved in a 9:1 mixture of chloroform:methanol to a final concentration of 2 mg/ml. Rho-DPPE was added to lipids in the rbf prior to removal of the solvents – a final Rho-DPPE concentration of 1 mol % was used based on work by Zelphati (Zelphati et al., 1998).

2.3 Characterisation of Liposomes

2.3.1 Vesicle size and zeta potential

Liposome vesicle size and zeta potential (ZP) were measured using a Brookhaven ZetaPlus instrument. The Brookhaven ZetaPlus measures the diameter of vesicles using the dynamic light scattering technique (DLS). This involves detection of scattered light from particles suspended in an aqueous solution. Particle movement, governed by Brownian motion, results in time-dependent variations in the light scattered which is detected and transformed into a linear measurement by way of the Stokes-Einstein equation. DLS is dependent on numerous factors including the concentration of the substance (in this case liposomes) being measured, the ionic strength of the aqueous solution used to suspend the liposomes, and the temperature. In order to limit variability, all liposomes were measured in a 10-fold dilution of the same buffer used for their production. Liposomes which had been stored at 4 °C were bought to room temperature prior to measurements.

In preliminary experiments it was decided that 100 µl of liposomes (1.25 mg/ml) and therefore equivalent to approximately 125 µg lipid was sufficient for accurate determination of the vesicle size and ZP. Therefore for all size and ZP studies, 100 µl of liposomes (1.25 mg/ml) were added to approximately 3 mls of hydration buffer (10-fold diluted) and mixed well. In addition to the overall polydispersity of the sample, three size measurements were recorded from each run. Therefore, unless stated otherwise, triplicate experiments resulted in a mean vesicle size derived from 9 values. Values which were clearly anomalies or outliners were not included. To measure the ZP a palladium probe (Brookhaven Instruments) was inserted into the sample (the same one used to measure vesicle size) and 10 measurements recorded per sample. Again, values which were clearly anomalies were not included. The mean result of triplicate experiments was therefore derived from a maximum of 30 values.

2.3.2 Imaging of liposomes

A 1 ml sample of liposomes (1.25 mg/ml) was placed in a small clear HPLC vial and stored under the appropriate conditions. Photos were taken of the samples at various time-points to compare liposome formulation stability.

2.3.2.1 Transmission electron microscopy (TEM)

TEM was conducted at The University of Birmingham with the help of Paul Stanley. Liposomes were produced as described in section 2.2.1. A small drop (~ 10 µl) of liposomes was placed onto a 3.05 mm copper grid containing numerous hexagonal pores. The sample was left for 30 seconds and then blotted to remove excess solution. A 10 µl sample of uranyl acetate (UA) stain was placed onto the grid and again blotted after 30 seconds. The sample was viewed with a transmission electron microscope and photos taken. In addition to UA stain, ammonium molybdate (AM) also known as ammonium heptamolybdate ($(NH_4)6Mo_7O_{24}$), and phosphotungstic acid (PTA; $H_3PW_{12}O_{40}$) were also investigated as AM and PTA have both been described to work best at neutral pH values. In contrast UA works best at pH 4 and is known to precipitate at higher pH values. All three are negative stains that dispose heavy atoms on the surface of the samples – one of the consequences can be the flattening of larger liposomal structures. Comparative images of DDA:TDB liposomes stained with all three stains were taken.

2.3.2.2 Fluorescence imaging of liposomes

Fluorescently labelled liposomes were produced as described in section 2.2.3 with the inclusion of Rho-DPPE which allows liposomes to be viewed under the rhodamine filter on a fluorescence microscope. Occasionally liposomes were also viewed with the addition of fluorescein isothiocyanate (FITC) labelled ovalbumin (OVA). To view the samples a small drop (~ 10 µl) of liposomes was placed on a standard glass microscope slide and covered with a glass cover slip. Liposomes were viewed using phase contrast and fluorescence filters (Rhodamine for liposomes, FITC for protein).

2.4 Methods in Protein Detection

2.4.1 The BCA assay

For simple, quick and accurate protein detection and quantification, the bicinchoninic acid (BCA) protein assay was used. The assay relies on reduction of Cu^{2+} to Cu^{1+} which is proportional to the amount of protein present. Analysis is based on a colour change from green to purple/blue which is detected at 562 nm. To measure the presence of protein accurately the protein concentration should be between 0.2 – 1 mg/ml which relates to

protein concentrations of 5 – 25 µg/25 µl (the suggested volume for use in a 96-well plate). To make the BCA reagent, a 50:1 ratio of components A and B are mixed; 200 µl of this working reagent is added to the protein-containing wells of a 96-well plate. The absorbance, read at 562 nm, can be translated into a protein concentration with the aid of a non-linear polynomial standard curve produced using a serially diluted 1 mg/ml protein stock (Pierce Biotechnology).

Determination of protein adsorption to liposomes was undertaken indirectly by measuring the protein presence in the supernatant of centrifuged liposomes as opposed to that adsorbed directly to the liposome. This is due to the lipid content of the liposome suspension interfering with the absorbance readout.

2.4.2 SDS-PAGE

Gel electrophoresis was also used to semi-quantify protein adsorbed to liposomes and/or found unassociated. A standard gel electrophoresis protocol was followed using Tris-glycine gels and staining either with coomassie blue or silver stain. The silver staining technique is known to be 10-50 fold more sensitive for protein detection than coomassie staining and was therefore used when the protein levels were considered especially low (Merril et al., 1981). Silver staining was conducted with a Bio-Rad kit using the standard staining procedure outlined in the protocol. Briefly, once the electrophoresis run was complete, the gels were removed, fixed, oxidized, washed and silver reagent (silver nitrate) added, followed by a further wash and then development using a pre-mixed sodium carbonate/paraformaldehyde solution. A 5 % (v/v) acetic acid solution was used to stop development.

2.4.3 Radiolabelling of proteins

In order to detect protein in biodistribution studies, radiolabelling was implemented using ^{125}I. Iodination of protein was undertaken using Pierce iodination tubes containing Pierce iodination reagent (formally known as IODO-GEN® tubes) which contain an oxidizing reagent that converts $Na^{125}I$ into a reactive iodine molecule that can insert into the tyrosol group of tyrosine amino acids (Figure 2.1). The number of tyrosine amino acids in the protein to be labelled is therefore of importance, however for proteins which contain few tyrosine amino acids the Bolton-Hunter reagents can be used to introduce iodinatable

sites. The amount of ^{125}I added to the iodination tube was dependent on the type of protein being labelled and the amount of protein required. For the labelling of OVA and Ag85B-ESAT-6, a pH 7.4 Tris buffer (10 mM) was used as the proteins were stable in these conditions. OVA and Ag85B-ESAT-6 contain 9 and 13 tyrosine amino acids respectively; this was sufficient for efficient radiolabelling. For the radiolabelling of lysozyme, Tris buffer (10 mM) was also used however the pH was reduced to pH 4 with the use of HCl as preliminary studies showed this pH gave the best detection of unlabelled lysozyme using the BCA assay. The protein CTH1 was radiolabelled in a pH 4 phosphate citrate (PC) buffer (70 mM) as it was supplied and is stable in this buffer. For both *in vitro* stability studies and *in vivo* biodistribution studies each dose contained ~ 100 kBq of ^{125}I. For *in vivo* studies mice received a dose of 2 µg Ag85B-ESAT-6 or 5 µg CTH1 or lysozyme. Therefore the protein:^{125}I ratio varied, as did the radiolabelling efficiency for the different proteins. Furthermore, as the decay rate of ^{125}I is 60 days the volume of ^{125}I required was variable. Sample volumes for the production of 40 doses of protein (2 µg/dose) would therefore involve the addition of 100 µl protein (800 µg/ml) with 5.71 MBq ^{125}I (40 doses at 100 kBq each, plus 30 % extra due to labelling inefficiency and is therefore counted as 'free unbound' ^{125}I). For the radiolabelling of CTH1 and lysozome, additional ^{125}I was added to the iodination tube as the labelling efficiency decreases with each decrease in pH unit (Pause et al., 1982). For total volumes (^{125}I and protein) of over 200 µl, two iodination tubes were used to maximise iodination reagent:protein: ^{125}I interaction. The mixture was left in the iodination tube for between 45 – 90 minutes with frequent swirling to ensure complete exposure of protein and ^{125}I to the iodination reagent.

In order to remove unbound ^{125}I from the protein solution, a gel filtration column prepared with Sephadex™ G-75 was used. A glass 5 ml pipette was used to minimise protein/plastic interactions. Sephadex™ G-75, rehydrated with a 10-fold dilution of the same buffer used to dissolve the antigen in, was poured into the glass pipette leaving ~ 2 cm space at the top of the column (cotton wool was used to block the bottom of the pipette). The total volume of the column was therefore ~ 8 cm^3 and the elution speed was ~ 0.25 ml/minute. Each minute a sample was collected which therefore resulted in the total column volume being eluted within 40 minutes. Frequent addition of the same buffer used to make the column was used to prevent the column running dry. Once 40 samples had been collected the column was disposed of and all the samples collected were counted on a γ-counter to quantify ^{125}I presence. To determine the presence of protein, 25

µl of each sample was removed and added to the wells of a 96-well plate; 200 µl of BCA reagent was added to each well and the plate was incubated at 37 °C for 30 min followed by reading the absorbance at 562 nm (see section 2.4.1 for more detail of the BCA assay). Samples which correlated for the presence of protein and ^{125}I were pooled; on average this accounted to four samples and therefore the resulting total volume of ^{125}I:protein was ~ 1 ml. This sample was diluted accordingly for *in vitro* stability or *in vivo* biodistribution studies.

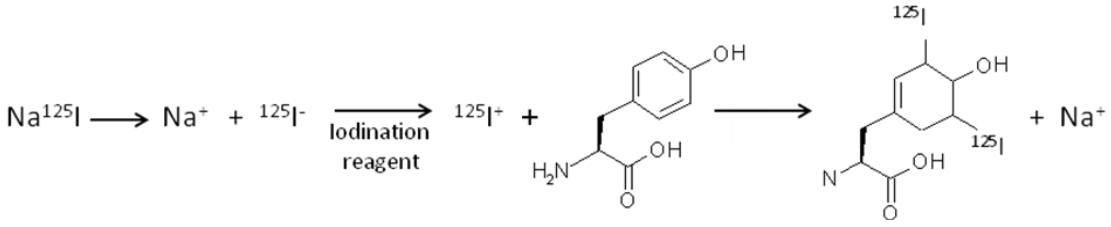

Figure 2.1 Diagram showing the incorporation of ^{125}I into amino acid residues of proteins during the radiolabelling procedure. The reaction was undertaken in Pierce iodination tubes containing Iodination reagent.

2.4.4 Fluorescence labelling of OVA protein

The model protein OVA was used to investigate protein binding to DDA:TDB liposomes using fluorescence techniques. OVA was labelled with FITC in a PBS buffer as opposed to Tris buffer due to the incompatibilities between amine-containing compounds with FITC. A 5:1 weight ratio of OVA:FITC was dissolved in PBS (10 mM, pH 7.4) to a final OVA concentration of 50 mg/ml. This was left overnight at 4°C in the dark with frequent swirling. A Sephadex™ G-75 gel filtration column was used to separate unbound FITC; the same method described in section 2.4.3 to remove unbound ^{125}I was implemented. The protein was eluted with 1 mM PBS buffer and the final concentration of OVA adjusted to 10 µg/ml.

2.5 Methods in Cell Culture

2.5.1 Culture and maintenance of continuous cell lines

Murine and human monocyte/macrophage like cell lines were used to investigate the effect of liposomes on cellular properties. The murine RAW 264.7 gamma NO(-) and J774.1 cell lines, both originally derived from the ATCC, were cultured in DMEM media supplemented with 10 % foetal bovine serum (FBS) and 1 % (penicillin-streptomycin-

glutamine (PSG). Cells grew as an adherent monolayer and were passaged by scrapping. Although a preliminary experiment showed that cell viability was increased if EDTA/trypsin was used as an alternative to scrapping, over time the ability of the cells to adhere was reduced, therefore scrapeing was used to dislodge cells. The cells grew quickly and required approximately 2-3 passages/week when passaged at a 1:9 ratio of cells:media. Cells were used between passages 7-20 however during early 2010 it seems likely that the liquid N_2 storage tank ran dry which forced us to throw out all cyropreserved vials. Consequently a new batch of J774.1 cells were obtained from a neighbouring lab and these cells were used between passage number 20-30. Low passage number was maintained by cyropreserving cells in 10 % dimethyl sulphoxide (DMSO) in FBS in a liquid N_2 tank. The human monocyte cell line THP-1, donated by Andrew Devitt, Aston University, Birmingham, was maintained in RPMI media supplemented with 10 % FBS and 1 % PSG. In certain studies this cell line was grown with the addition of vitamin D3 (VD3) to induce macrophage like properties. In such instances, VD3 was added to complete RPMI at a final concentration of 250 nM and cells typically differentiated within 2 days. All cell lines were maintained at 37 °C, 5 % CO_2 and 95 % relative humidity.

2.5.2 Determination of cell number

During routine cell passage, the dilution factor was estimated from the level of confluence. Determination of cell number for experiments or freezing was achieved via tryphan blue exclusion whereby 20 µl of resuspended cells was removed and mixed with an equal volume of tryphan blue. With the aid of a hemocytometer, cells excluding tryphan blue were quantified and the number of cells/ml was calculated using the following equation:

No of cells/ml = no. cells/sq x dilution factor x 10^4

Where: no. cells/sq is the average of 10 squares in the hemocytometer

Dilution factor is 2 if equal volumes of resuspended cells and tryphan blue are used

10^4 is the multiplication factor related to the volume of the hemocytometer grid

2.5.3 Measurement of cell characteristics

2.5.3.1 Neutral red assay

The neutral red (NR) assay was used as a simple method to determine cell viability. The method relies on the uptake of the viable stain 'neutral red' which becomes blocked in lysosomes of cells with an active metabolism. After washing of cell monolayers, the cells are lysed to release neutral red which can then by quantified using a spectrophotometer. In general, cells were seeded into a 96-well microplate at a density of 1-2 x 10^5 cells /ml with 100 µl cells/well and allowed to grow for 24-48 hrs or until ~ 70 % confluent. The NR assay was used to investigate cell viability as a consequence of liposome toxicity. Therefore, liposomes were added to cells at a range of concentrations (0.005 - 50µg lipid/ml) in media without the presence of FBS to prevent serum protein induced aggregation of liposomes. Liposomes were left on the cells for between 1 - 48 hrs followed by washing with cold FBS-free media and addition of 100 µl NR (100 µg/ml). After 1 hr cells were washed twice with cold PBS followed by addition of 100 µl 'stop solution' (1 % acetic acid:49 % H_2O:50 % ethanol) to lyse cells. Plates were gently tapped/swirled to ensure all cells were lysed and NR evenly distributed throughout the well. The absorbance at 405 nm was read to determine the proportion of viable cells compared to negative (lysis of cells using Triton-X prior to NR addition) and/or positive controls (cells without liposomes).

2.5.3.2 MTS assay

The MTS assay is a commonly used version of the previous MTT assay (and similar XTT assay) which all measure cell viability/proliferation/cytotoxicity in a colorimetric manner. The method relies on the cells ability to reduce 'MTS' into a soluble formazan product which can be measured at 490 nm using a spectrophotometer. Cells were treated in the same method as described for the NR assay (section 2.5.3.1) with the exception that instead of adding 100 µl NR, 100 µl of MTS reagent (20 % v/v with media) was added. The cells were left at 37 °C for between 1 – 4 hrs followed by measurement of the formazan product at 490 nm. The percentage of viable cells was determined against negative (lysis of cells using Triton X-100 prior to MTS addition) and/or a positive controls (cells without liposomes).

The MTS assay was used in initial experiments however the results produced were similar to the NR assay – ultimately the NR assay was used preferentially as it is cheaper and therefore a larger proportion of variables could be screened with ease. However, it is important to note that whilst the MTS assay relies on cells being metabolically active (as MTS is taken up by mitochondria), the NR assay relies on NR uptake and entrapment in lysosomes and therefore does not require cells to be metabolically active. For the purpose of cell viability due to liposome induced toxicity, the NR assay was sufficient.

2.5.3.3 LDH assay

In a similar method to the MTS and NR assays, the LDH assay can be used to give an indication of cell viability due to the toxic effects of liposomes (or indeed other factors). Unlike the MTS or NR assays which measure the number of viable cells, the LDH assay measures the number of damaged or lysed cells by detecting the presence of the intracellular enzyme lactate dehydrogenase (LDH) in the surrounding culture media. The enzyme LDH catalyses one of the two reactions which leads to formazan production which is subsequently detected using a spectrophotometer at 490 nm. Formazan production is directly proportional to the number of lysed cells. The process of LDH detection involved the same cell/liposome preparation and incubations as applied to the MTS and NR assays. As the supernatant and not the cells were assayed, at the end of the cell and liposome incubation period, 50 µl of culture supernatant was removed and placed into a new 96-well plate. To this, 50 µl of LDH substrate mix was added and the plates incubated at 37 °C for 30 min in the dark. The reaction was stopped by adding 50 µl of 'Stop solution' and the absorbance read at 490 nm. Numerous controls were included: media only ('background'), cells without liposomes (spontaneous LDH release), lysed cells (maximum LDH release) and a kit supplied LDH positive control to ensure the enzymatic reaction functions correctly.

2.5.3.4 NAG assay

NAG, or β-N-acetylglucosaminidase, is an enzyme found in mammalian cells which catalyses the hydrolysis of 4-nitrophenyl N-acetyl-β-D-glycosaminde (NP-GlcNAc) resulting in p-nitrophenol. This reaction is important for glycolipid and glycoprotein degradation in lysosomes and can therefore be used as a method to investigate cellular activity. The assay

was conducted using a kit whereby the provided substrate NP-GlcNAc is hydrolysed by cellular NAG. As NAG is an intracellular enzyme, cell lysis to release NAG into the culture supernatant for assaying is crucial. However, it should be noted that the NAG assay can also give false positives when looking for cellular activation if, for example, the cell has been lysed due to the toxicity of the components added. Therefore, it is wise to carry out a simultaneous cell viability assay (MTS, NR or LDH) to verify that the cell numbers are the same for all conditions.

The cells were treated with the relevant liposome formulations for the chosen time period. In some experiments cells were pre-treated with LPS to stimulate them. Media from all wells was removed and the cells lysed by adding 1 % Triton X-100. After 1 hr, 20 µl of cell supernatant was removed and added to a new 96-well plate. 80 µl of NAG substrate (Np-GlcNAc) was added to each well and the plate incubated for 30 min at 37 °C. 'Stop solution' (200 µl/well) was added and the absorbance at 405 nm was read using a spectrophotometer. Controls included unlysed cells (background NAG levels), substrate without cell lysate (non-specific NAG enzyme activity), colour control (maximum colour production) and an enzyme control using kit NAG enzyme and substrate (to confirm NAG substrate specificity).

2.6 Biodistribution Studies in Mice

Balb/c mice were used to study the movement of liposomes and antigen from the site of injection (SOI). Three to five mice were used for each experimental group: whilst 3 mice/group were used for the initial experiment, and 5 mice/group in the second experiment, it was finally decided that 4 mice/group was sufficient to produce quality data even in the presence of an outliner. Mice were housed under conventional conditions (22 °C, 55 % humidity, 12 h day/night cycle) in their experimental groups (therefore between 3-5 mice/cage) and were given a standard diet ad-lib. All mice were purchased at 6-8 weeks of age at the start of the experiment.

2.6.1 Pontamine blue for the detection of immune tissues

Four to seven days prior to injection of the vaccines, sterile filtered (0.2 µm) pontamine blue (0.5 % w/v in PBS) was injected subcutaneously (s.c) into the neck region as a marker for lymph nodes (Tilney, 1971). The volume injected (100 - 200 µl) depended on the

mouse weight/age but was always standardized within each experiment. In certain biodistribution experiments a tail-bleed (50 µl) was taken 3 days after injection of pontamine to identify cell types taking up pontamine blue. To collect blood, capillary tubes lightly coated in 1 % heparin (in PBS) were used to draw up blood from a small incision at the base of the tail. The blood from 20 mice was pooled in PBS (10 mM) and washed once (13,000 rpm, 5 min). OptiLyse®C lysing solution (1 ml) was added and left for 10 min followed by addition of 1 ml PBS (10 mM). The samples were mounted onto glass slides using a cytospin and mounted with glass cover slips using a DAPI-containing hard mount solution (Vector labs). Samples were viewed using a fluorescent microscope to identify pontamine blue containing cells (the fluorescent nature of pontamine blue is attributed to its six benzene rings).

2.6.2 Preparation of radiolabelled vaccines

Biodistribution studies were conducted with ^{3}H-labelled liposomes and ^{125}I-labelled protein antigen, produced as described in sections 2.2.2 and 2.4.3 respectively. Due to the dilution of antigen during the gel filtration step after iodination, the concentration of the liposomes had to be increased 2-fold so that upon mixing of equal volumes of protein and liposome, the final concentration remained 1.25 mg lipid/ml equivalent to 250 µg lipid/dose. Furthermore, the final concentration of the liposomes and antigen had to be further concentrated as whilst this dosage volume (200 µl) is suitable for subcutaneous, intraperitoneal (i.p) or intravenous (i.v) injections, the recommended volume for intramuscular (i.m) injection is 50 µl. Therefore, liposomes were actually rehydrated in a volume of buffer 8-fold less than usual, followed by addition of an equal volume of antigen at a concentration 2-fold higher than required. Table 2.2 summarizes these volumes with respect to DDA:TDB liposomes. Furthermore, as Tris buffer used to rehydrate lipid films is not isotonic, 20 % (w/v) trehalose was included in the Tris buffer so that upon dilution with an equal volume of radiolabelled protein, the final trehalose concentration was 10 % (w/v). Approximately 1 hr prior to injection, liposome and antigen were mixed; each dose contained 250 µg lipid, 50 µg TDB and 2 or 5 µg protein antigen. Vaccines were given by either the i.m route into the quadriceps (dose volume 50 µl) or via the s.c route into the flank (dose volume 200 µl).

Table 2.2 Quantity of liposome components required to make 'DDA:TDB' liposomes for a range of experiments

Application	DDA (μg)	TDB (μg)	Rehydration buffer (μl)	Volume of antigen required (μl)
Physicochemical analysis	1250	250	1000[1]	
BD study (s.c)	250	50	100	100
BD study (i.m)	250	50	25	25
BD study (i.m) 'Big liposomes'	250	50	10[2]	25

BD, biodistribution; s.c, subcutaneous; i.m, intramuscular
[1] Assuming no antigen will be added.
[2] With a further 15 μl buffer added once liposomes have formed.

2.6.3 Processing of tissues

Mice were terminated at the relevant time-points by cervical dislocation and various tissues collected (Table 2.3). The exception to this was for experiments in which the blood was also collected; in this case blood was collected by cardiac puncture with mice asphyxiated by CO_2, followed by cervical dislocation to confirm death. All tissues were processed in the same manner, the major steps being tissue solubilisation, quantification of ^{125}I, bleaching followed by quantification of ^{3}H. Each tissue sample was weighed into a γ-vial and Solvable™ (1.5 ml) added to each sample before quantifying ^{125}I presence using a Cobra™ CPM Auto-Gamma® counter (Packard Instruments Company inc., IL, USA). To verify that the presence of undigested tissue and whole organs did not affect the count rate, all samples from original experiments were counted for ^{125}I prior and post tissue digestion with no difference in the results noted (results not shown). Samples were heated at 40 °C until tissues were fully solubilised, following which they were allowed to cool and then transferred into 20 ml scintillation vials. Both glass and plastic scintillation vials were used although within each experiment the vial type was kept constant. Hydrogen peroxide (200 μl) was added to each sample and once fully bleached (sometimes additional heating of samples was required to ensure complete digestion), Ultima Gold™ scintillation fluid (10 ml) was added to each sample and ^{3}H presence quantified using a standard ^{3}H detection protocol.

Table 2.3 Approximate weights of various tissues collected during biodistribution studies

Tissue	Approximate weight collected (mg)
Blood	100
Heart*	150
Lung	100
Liver	100 - 200
Kidney *(one of two)	150
Spleen*	100
Small Intestine	100
Brain	100 - 200
Site of injection (quadriceps)	300 - 400
Site of injection (flank)	200 - 300
Lymph node* (local draining)	1 - 2

Not all tissues were collected in every study. * indicates whole organ

2.6.4 Quantification of the proportion of vaccine components in tissues

For the determination of liposome (^{3}H) and antigen (^{125}I) in the different tissues, the data was either presented as radioactivity (counts per minute; CPM) or a proportion of the dose (% dose). In both cases, the results were presented as whole tissue or mg tissue. To calculate the % dose, triplicate samples of the original dose were processed simultaneously to the tissue processing. These doses represented a fraction of the whole dose and relevant dilution factors were considered after obtaining the CPM values. For all samples, the CPM values derived from the Cobra™ CPM Auto-Gamma® counter (relating to ^{125}I presence) did not need additional processing as ^{3}H cannot be detected by the gamma counter without the presence of a scintillant; solely the removal of background values (average of three Solvable™ samples) was undertaken. For the determination of ^{3}H however the ^{125}I overspill had to be considered as ^{125}I is detectable on a scintillation counter. To overcome this problem a method was developed whereby the ^{125}I could be factored out by the use of a standard curve. Triplicate samples of ^{125}I starting at an activity equivalent to the dose administered *in vivo* (~ 100 kBq) were diluted 2-fold until background levels (~ 20 cpms) were reached. These samples were counted using a γ-counter and then transferred and processed as the tissue samples would be (see section 2.6.3). The samples were then counted on a scintillation counter using the same ^{3}H detection protocol as used for the tissue samples. A plot of the cpm values derived from the gamma counter (x-axis) against the cpm values derived from the scintillation counter (y-axis) was made and the line of best fit and equation derived for samples below 50,000 cpm (~ 2 % of the dose or less) and those above 50,000 cpm (~ 2 % of the dose or more).

These two equations were used to calculate the effective interference that the ^{125}I would have on the ^{3}H values determined by scintillation counting. We found that the efficiency of ^{125}I counting using our scintillation counters was approximately half of that seen by the γ-counter; however for each separate biodistribution study conducted, a new ^{125}I standard curve was produced as the counting efficacy of the machine can change and is also dependent on the amount of ^{125}I in the sample. To investigate whether this relationship was linear, known volumes of ^{3}H were added to the serially diluted ^{125}I samples and counted on the scintillation counter. The results showed a linear relationship indicating that there is no quenching effect between the two radioisotopes and that the above equation method to remove interfering ^{125}I counts is valid.

2.7 Immunisation Studies in Mice

2.7.1 Experimental set-up

Two separate immunisation studies were conducted, the first in partnership with lab colleagues (RK, Randip Kaur; JH, Jubair Hussain; MH, Malou Henriksen) and the second conducted alone by MH. The number of mice per experimental group and allocated per researcher was not constant between experiments – Table 2.4 outlines the experimental design.

Table 2.4 Outline of the liposome formulations used in the two immunisation studies

	Immunisation study	
Formulation	1	2
1	DDA:TDB:OVA (6)	DDA:TDB:H1, Small (5)
2	OVA (6)	DDA:TDB:H1, Medium (5)
3	OVA with ICF (3)	DDA:TDB:H1, Large (5)
4	PBS (3)	DDA:TDB:H1, Extra large (5)
5		H1 with ICF (4)
6		PBS (4)

Numbers of mice per group are shown in brackets. H1, Ag85B-ESAT-6

2.7.2 Vaccine formulations and preparation

Liposomes were produced as described in section 2.2.1 with the addition of 10 % trehalose to the rehydrating Tris buffer to provide isotonicity. Furthermore, the volume of Tris used

for rehydration was adjusted so that upon addition of antigen (OVA or Ag85B-ESAT-6) the final dose (50 µl) contained 250 µg DDA, 50 µg TDB and 10 µg OVA or 2 µg Ag85B-ESAT 6.

For the OVA immunisation experiment, injection of OVA alone was used to determine the adjuvantal effect of DDA:TDB liposomes. In this case OVA was dissolved in PBS (10 mM, pH 7.4) to a final concentration of 200 µg/ml so that each dose (50 µl) contained 10 µg OVA.

For the Ag85B-ESAT-6 immunisation experiment, the aim of the experiment was to determine the effect of vesicle size on resulting immunogenicity. Ag85B-ESAT-6 adsorbing DDA:TDB liposomes were made to four different sizes. All formulations were made using the lipid-film hydration method (see section 2.2.1) with the following described alterations. To produce small liposomes the rehydrated liposome mixture was sonicated at 4 microns for 2 x 30 seconds. Medium sized liposomes were not altered in any way. Large liposomes were produced by rehydration of the lipid film in a volume equal to one fifth of the required volume. Once cooled, the remaining four fifths of the required rehydration volume was added. To produce the largest liposomes, PBS buffer (10 mM, pH 7.4) rather than Tris buffer was used as the rehydrating medium. As PBS is isotonic, no trehalose was added to the buffer unlike the small, medium and large liposomes which were all rehydrated in Tris buffer containing 10 % trehalose.

Both studies included a positive (antigen with ICF) and negative (PBS buffer alone) control. For the production of the antigen:ICF positive control, equal volumes of antigen (OVA or Ag85B-ESAT-6) and ICF were vortexed well until a homogenous solution resulted. Each dose (50 µl) contained 10 µg OVA or 2 µg Ag85B-ESAT-6 in combination with 25 µl ICF. The negative naïve control group was PBS alone for both experiments. Mice were injected i.m (50 µl) into the left or right quadriceps with formulations prepared on the day of injection.

2.7.3 Antibody analysis

Antibodies were detected in the serum of tail-bled mice. Blood was collected using capillary tubes dipped in 1 % heparin (PBS, 10 mM) and placed immediately into eppendorfs containing 450 µl PBS (10 mM). All tubes were centrifuged (13,000 x g, 5 minutes) and the supernatant from each tube was placed in a new eppendorf and frozen (-20 °C) until use. Serum was consequently 20-fold diluted in PBS if the volume ratio of red blood cells:sera is assumed to be 1:1.

To detect antibodies in serum, standard enzyme-linked immunosorbent assays (ELISA) were used. ELISA pates were coated overnight at 4 °C with 50 µl OVA in PBS (2 µg/well) or Ag85B-ESAT-6 (5 µg/ml) for the relative studies (Agger et al., 2008b). Plates were washed three times to remove unbound antigen; the wash buffer used throughout was 'PBST' (40 g NaCl, 1 g KCl, 1 g KH_2PO_4, 7.2 g $Na_2HPO_4(2H_2O)$, 0.4 ml Tween 20, 5 L ddH_2O). Marvel milk powder (100 µl; 1 % w/v) was added per well to block non-specific binding (Agger et al., 2008b). After 1 hr incubation at 37 °C, the plates were washed three times with PBST and were now ready for addition of serially diluted serum. Figure 2.2 shows an example plate setup for the OVA immunisation study: 190 µl of PBS (10 mM, pH 7.4) was added to each well in row A whilst 100 µl PBS was added to each well in rows B-H. Serum (10 µl) was added to the appropriate wells of row A and mixed well – a 50 µl sample was removed and added to the wells in row B. Again, this was thoroughly mixed and the serial dilutions continued until lastly 50 µl of diluted serum was removed from the wells of row A and H so that the total volume of serially diluted serum in all wells was 100 µl. Each sample was investigated in duplicate. Plates were incubated at 37 °C for 2 hrs followed by three washes with PBST and addition of 50 µl of isotype specific immunoglobulin (IgG, 1:500; IgG1, IgG2a, IgG2b, 1/4000 dilution). After 1 hr at 37 °C all plates were washed again three times and 50 µl of substrate added per well. The substrate comprised 5 x 10 mg tablets of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) dissolved in 83.3 ml citrate buffer (0.92 g citric acid, 1.956 g Na_2HPO_4, 100 ml ddH_2O) and 7.5 µl H_2O_2. After 20 min incubation at 37 °C, the absorbance at 405 nm of all wells was measured (Bio-Rad, model 680). The results were expressed as the $\log_{10}$ value of the reciprocal of the end point dilution which gave an optical density (O.D) of 0.2 or above.

		1	2	3	4	5	6	7	8	9	10	11	12
		1.1	1.2	1.3	1.4	1.5	1.6	1.1	1.2	1.3	1.4	1.5	1.6
A	1/400												
B	1/1,200												
C	1/3,600												
D	1/10,800												
E	1/32,400												
F	1/97,200												
G	1/291,600												
H	1/874,800												

Figure 2.2 Outline of the ELISA plate set-up used for the OVA immunisation study to study the adjuvant ability of DDA:TDB liposomes in OVA immunisation.

2.7.4 Splenocyte proliferation

To test cells for their ability to respond to antigen *in vitro*, splenocytes were restimulated with various concentrations of antigen and their proliferation, determined by ^{3}H-thymidine uptake, measured. On day 49 mice were terminated by cervical dislocation and their spleens harvested and placed in a 7 ml bijoux containing ~ 5 ml ice cold PBS. Each spleen was treated individually and kept on ice until processing. Spleens were mashed through a cell strainer into a 50 ml centrifuge tube using the 5 ml PBS and a further 10 ml cold cRPMI (RPMI containing 10 % FBS and 1 % PSG). Each tube was left for ~ 5 min to allow cell debris to settle. The supernatant (~ 13 ml) was transferred into a 15 ml falcon tube and centrifuged at 1200 rpm, 15 °C for 10 min, after which the supernatant was discarded and the pellet resuspended in 10 ml cold cRPMI. The wash was repeated and the pellet resuspended in 5 ml cold cRPMI and a cell count performed as described in section 2.5.2. The cell number was adjusted to between 0.2-1 x 10^{7} cells/ml.

According to the immunisation experiment, serial dilutions of OVA (0.5, 5 and 50 µg/ml) or Ag85B-ESAT-6 (0.05, 0.5 and 5 µg/ml) in cRPMI were made and 100 µl added per well of a 96-well culture plate (Holten-Andersen et al., 2004). Wells containing medium only or 5 µg/ml of concanavalin A (ConA) were included in all experiments as negative and positive controls respectively. Splenocytes (100 µl, 0.2-1 x 10^{7} cells/ml) were added to each well making a final well volume of 200 µl. Cultures were incubated at 37 °C, 5 % CO_2, 95 % humidity for 72 hrs following which 18.5 kBq (0.5 µCi) ^{3}H-thymidine (40 µl in cRPMI/well) was added (Eyles et al., 2003). After a further 24 hrs incubation under the same conditions, cells were harvested using a cell harvester (Titertek). For harvesting, media and cells from each well was aspirated onto a quartz filter mat. Each mat was placed into a plastic scintillation vial and 5 ml Ultima Gold™ scintillation fluid added/sample. All samples were counted using a standard ^{3}H scintillation counting protocol.

2.7.5 Cytokine analysis from in vitro restimulated splenocytes

Splenocytes were prepared in the same way as described in section 2.7.3. According to the immunisation experiment, dilutions of OVA (5 and 50 µg/ml) or Ag85B-ESAT-6 (0.05, 0.5 and 5 µg/ml) in cRPMI were made and 100 µl was added per well of a 96-well culture plate. Wells containing medium only or 5 µg/ml of ConA were included in all experiments as negative and positive controls respectively. Six to ten wells for each condition were

used so that sufficient supernatant could be obtained. Splenocytes (100 µl, 0.2-1 x 10^7 cells/ml) were added to each well and the plates incubated at 37 °C, 5 % CO_2, 95 % humidity for 48 hrs, following which the supernatants were removed, pooled according to group and frozen at -70 °C until use. Supernatants were assayed for the presence of IFN-γ, IL-2, IL-5, IL-6 and IL-10, using standard duo-set capture ELISA. Whilst the protocol was the same for each ELISA, there were variations in the concentrations of ELISA components. The general setup involved coating ELISA 96-well microplates overnight at rtp with 100 µl of the appropriate capture antigen (IL-2 and IL-5, 1 µg/ml; IL-6, 2 µg/ml; IL-10 and IFN-γ, 4 µg/ml). The following morning plates were washed using PBST (for recipe see section 2.7.3) followed by a block step with 300 µl of 1 % BSA in PBS containing 0.05 % NaN_3. After approximately 1 hr at rtp, the plates were washed again using PBST and the samples and standards added (100 µl/well). The cytokine standards were 2-fold serial dilutions of the supplied cytokine diluted in 0.2 µm sterile filtered reagent diluent (0.1 % BSA, 0.05 % Tween 20 in Tris buffered saline (20 mM Tris, 150 mM NaCl), pH 7.2 – 7.4). Each standard was diluted 6 times and included a 0 pg/ml control. Whilst most samples were added neat without any dilution, where necessary reagent diluents was used to dilute samples and dilution factors applied correspondingly. After 2 hrs incubation at rtp, the plates were washed again in PBST and 100 µl of the appropriate detection antibody added. New microplate slips were added to each plate and following a further 2 hrs incubation at rtp the plates were washed again with PBST. Streptavidin-HRP conjugate, diluted in reagent diluents, was added to each well (100 µl) and plates incubated in the dark at rtp for 20 min. Following a further wash with PBST, a substrate solution produced of 1:1 ratio of component A (H_2O_2) and component B (tetramethylbenzidine (TMB) agent (1 mg tablet), with 1 tablet dissolved in 1 ml DMSO and 9 ml phosphate citrate buffer (0.05 M, pH 5)) was added to each well (100 µl) and again left in the dark for 20 min. The reaction was stopped by addition of 50 µl/well 'STOP' solution (2N H_2SO_4) and the absorbance (O.D) at 450 nm and 560 nm of each well read using a microplate reader (BioRad, model 680).

All standards and samples were assayed in duplicate and the O.D at 560nm removed from the 450nm value to correct for imperfections in the ELISA plates. The average values of each standard and sample were determined and the average 0 pg/ml cytokine standard value was subtracted from all other standards and samples. Using GraphPad Prism, a four-parameter sigmoidal standard curve was created for each set of cytokine standards and the cytokine concentrations in the unknown samples calculated accordingly.

2.8 Statistics

For all experiments the mean ± standard deviation (S.D) was calculated, unless stated otherwise. Statistical analysis of data was tested by one- or two- way analysis of variance (ANOVA). When significant differences were indicated, differences between means were determined by Bonferroni's multiple comparison tests. All statistical analyses were performed in GraphPad Prism version 4 or 5 (GraphPad Software Inc., La Jolla, CA).

2.9 Collaborative Works; brief methods

2.9.1 Trinity College Dublin, Ireland

To investigate the ability of liposomes to stimulate production of IL-1α and Il-1β from DCs, bone marrow-derived dendritic cells (BMDCs) from C3H/HeN mice were plated at 6.25 × 10^5 cells/ml and incubated for 6 hours in the presence of LPS (5 ng/ml). Cells were then stimulated with DDA, DDA:TDB or DSPC:TDB liposomes which were made at Aston University using the protocol described in section 2.2.1 to a final concentration of 5 mg lipid/ml. Liposomes were added to the cells at concentrations starting with a 10-fold dilution (500 µg/ml) and thereafter 4-fold dilutions until a dilution of 1/40,960 was reached. Supernatants were removed after a further 24 hours and analysed for IL-1α and IL-1β by ELISA. The results are presented as the mean of triplicate samples ± S.E.

2.9.2 Statens Serum Institute, Denmark

The collaboration with Statens Serum Institute (SSI) involved the shared aim of basically improving cationic liposomes formulations designed as adjuvants for immunisation against tuberculosis (TB). The TB antigen Ag85B-ESAT-6 used for work both at Aston University and SSI was supplied by SSI. In addition to the immunisation studies which took place at SSI, a TB challenge experiment also took place. In all cases, liposomes were made at Aston University using the method described in section 2.2.1. Liposomes were made so that each dose would contain 250 µg lipid +/- 50 µg TDB. Ag85B-ESAT-6 (2 µg/dose) was added prior to immunisation at SSI. Analytical methods including antibody and cytokine detection, T-cell polyfunctionality, T-cell cycling *in vivo*, dendritic cell uptake studies and TB challenge studies are all described in the respective papers (Christensen et al., 2010b; Henriksen-Lacey et al., 2009; Henriksen-Lacey et al., 2010b, c). This work was supported by projects

TBVAC (contract n. LSHP-CT-2003-503367) and NewTBVAC (contract no. HEALTH-F3-2009-241745).

2.9.3 University Hospital of Zurich, Switzerland

The importance of the route of injection and antigen delivery system was investigated in a collaboration involving Leiden/Amsterdam Centre for Drug Research (Netherlands), ETH Zurich (Switzerland), Aston University and The University Hospital of Zurich, the later being the location of the experiment. The experimental details are fully described in the relevant paper (Mohanan et al., 2010).

Chapter 3: The Role of Liposomes in the Successful Formation of an Antigen Depot

Papers relating to this chapter:

Henriksen-Lacey M, Bramwell V.W, Christensen D, Agger E.M, Andersen P and Perrie Y. Liposomes based on dimethyldioctadecylammonium promote a depot effect and enhance immunogenicity of soluble antigen. J Control Release, 2009, 142:2, 180-186.

Henriksen-Lacey M, Bramwell V.W and Perrie, Y. Radiolabelling of antigen and liposomes for vaccine biodistribution studies. Pharmaceutics, 2010, 2, 91-104.

Mohanan D, Slütter B , Henriksen-Lacey M, Jiskoot W, Bouwstra J , Perrie Y, Kündig T.M, Gander B and Johansen P. Administration routes affect the quality of immune responses: a cross-sectional evaluation of particulate antigen-delivery systems. J Control Release, 2010, 147:3, 342-349.

3.1 Aims

This chapter addresses the initial studies conducted on liposomes based on dimethyldioctadecylammonium bromide (DDA). The physicochemical effects of additional stabilising and immunomodulatory molecules, in addition to the choice of rehydrating buffer and its molarity, on the DDA based formulations are presented. The stability of these formulations is addressed and both model and antigenic proteins are investigated in combination with the different DDA based liposomes in immunisation studies. Furthermore the biodistribution of the vaccine constructs and the importance of injection route are presented.

3.2 Introduction

Nearly 50 years have passed since the lipid DDA was originally described as having immunogenic properties (Gall, 1966), however it is still clear that our understanding of its adjuvantal abilities are inconclusive. The adjuvant action of liposomes composed of DDA has been primarily attributed to its ability to protect and deliver antigen to APCs and to form what is termed as an 'antigen depot' whereby antigen is retained at the injection site for a prolonged period of time. Antigen protection is of great importance as the increasingly common antigenic recombinant peptides and proteins produced are small and non-immunogenic and consequently enzymatic breakdown or more simply clearance by the circulatory system results in rapid clearance, normally via the kidneys, of these important molecules. Liposomes are good antigen-protectors as they convert 'soluble' antigenic molecules into a particulate form, rendering them effectively larger and more immunogenic. This trait of adjuvants - to make soluble antigen into a particulate substance - is not a sole property of liposomes but is in fact a common property shared by all adjuvants including emulsions and aluminium salts among others. However the ability of various adjuvants to retain antigen in a particulate form varies significantly and can be due to numerous factors such as the administration route or simply adjuvant breakdown in *vivo.* In terms of the antigen depot-effect, this is a property which also appears to be shared across numerous adjuvants, in particular aluminium salts. However, aluminium salt adjuvants tend to produce granulomas due to tissue damage and uric acid production (Goto et al., 1997). It is also likely that the viscous nature of liposomes may also lead to an antigen (and liposome) depot-effect in injected tissues.

3.2.1 Improving DDA-based liposome stability

Liposomes composed of DDA have been described as having immunostimulatory properties in numerous TB studies (Andersen, 1994; Bosio and Orme, 1998; Brandt et al., 2000; Davidsen et al., 2005; Holten-Andersen et al., 2004; Lindblad et al., 1997), however, their poor stability and only weakly immunogenic responses led to investigations in which additional stabilising molecules and/or immunomodulators were incorporated. Cholesterol is an abundant component of mammalian cell membranes and has been extensively studied as an artificial membrane stabiliser using phospholipid liposomal systems (Gregoriadis and Davis, 1979; McMullen and McElhaney, 1996; Sulkowski et al., 2005). The incorporation of cholesterol into liposomal membranes has been shown to lead to improved lipid packing consequently reducing or even eliminating the main phase transition temperature (Christensen et al., 2009; Ohtake et al., 2005). The resulting lower gel to liquid phase transition temperature leads to an increased bilayer fluidity and liposomes show improved stability both *in vitro* and *in vivo* (Gregoriadis and Davis, 1979). In fact many of the earlier studies using liposomes as drug delivery systems incorporated cholesterol to not only improve liposome stability but also to increase the half-life of intravenously administered formulations; the inclusion of cholesterol into the liposome membrane limits plasma protein association with the liposome surface and consequently reduces the tendency of liposomes to be rapidly uptaken by the mononuclear phagocyte system (MPS) (Gregoriadis, 1985, 1995b; Semple et al., 1996). Whilst cholesterol is not inherently immunogenic, numerous studies have shown that incorporation of cholesterol into liposomes leads to more favourable liposome properties such as increased transfection rates (Xu and Anchordoquy, 2008) and improved immunogenicity (Bakouche and Gerlier, 1986). However this is not a rule of thumb for all liposome formulations as cholesterol inclusion in other liposome formulations has been shown to lead to reduced immunogenicity (Christensen et al., 2009; Nakano et al., 2002). Therefore whilst liposome stability can be improved by using cholesterol, this is not always the optimum method due to the forfeited loss of immunogenicity.

3.2.2 Methods to augment the immunostimulatory actions of DDA-based liposomes

In an attempt to improve the immunogenicity of DDA liposomes, the incorporation of various bacterial derived substances has been investigated. Perhaps the most common is

monophosphoryl lipid A (MPL), a less toxic version of lipid A which is the anchor moiety of Gram-negative bacteria lipopolysaccharide (LPS) (Alving and Rao, 2008). Addition of MPL to DDA liposomes has been shown to significantly improve the immunogenicity of the formulation with strong cell-mediated immune responses observed in TB studies (Brandt et al., 2000; Holten-Andersen et al., 2004; Langermans et al., 2005; Olsen et al., 2001). Two mycobacterial derived substances which have been studied in TB models include N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide; MDP), administered in solution (Pettis et al., 2000) and liposomal form (Holten-Andersen et al., 2004), and trehalose 6,6'-dimycolate (TDM). TDM has been used in a wide range of disease models as it is a strong inducer of humoral and cell-mediated immunity. Only recently has a cellular receptor for TDM (and its synthetic analogue) been discovered (Ishikawa et al., 2009) although the receptor itself was first identified over a decade ago (Matsumoto et al., 1999) and has been known as a receptor for certain fungal species (Wells et al., 2008; Yamasaki et al., 2009). The receptor, known as Mincle or CLEC4, is postulated to directly bind the TDM glucose head groups possibly in a 2:1 Mincle:TDM ratio, although this has yet to be fully tested (Ishikawa et al., 2009). The discovery of the Mincle receptor is important as a specific target is now identifiable rather than the previously considered scavenger receptor MARCO and also TLR 2 to which TDM was linked (Bowdish et al., 2009). Furthermore, in addition to the receptor itself, the downstream signalling pathway has been identified as the Syk-Card9-Bcl10-Malt1 pathway; using selective knock-out studies TDM and its synthetic form trehalose 6,6'-dibehenate (TDB) have been shown to signal via this pathway resulting in the induction of the innate immune response with a Th1/Th17 bias (Werninghaus et al., 2009).

TDM is a large molecule ($C_{186}H_{366}O_{17} \pm 10CH_2$; M_w 2970 ± 140) (Noll et al., 1956) and is known to interfere with lipid organisation in liposomal membranes (Crowe et al., 1994; Spargo et al., 1991). Furthermore the similarity between TDM and its natural pathogen makes it relatively toxic *in vivo* and it is therefore unsuitable for use in vaccines (Hunter et al., 2006; Kato, 1973). The synthetic analogue TDB is composed of the same glucose disaccharide head groups whilst the mycolate chains are reduced in number and their length shortened (Matsunaga and Moody, 2009) leading to a significantly decreased toxicity whilst retaining immunogenic properties. TDB is considerably smaller than its natural analogue (M_w 987) and can be readily incorporated into DDA liposomes at concentrations of at least 20 mol % without any significant changes to properties such as liposome size or IFN-γ production in immunisation studies (Davidsen et al., 2005). The

optimum amount of TDB incorporation into DDA liposomes is observed at 11 mol % and in addition to producing liposomes expressing long term stability at 4 °C (Linderstrøm et al., 2009), this concentration has been shown to give the highest antibody titres when compared to other TDB concentrations (Davidsen et al., 2005). TDB has also been shown to be successfully incorporated into DDA lipid bilayers up to a 1:1 TDB:DDA w/w ratio (equivalent to 39 mol % TDB) and although the membrane fluidity of the liposomal systems decreases, the main gel to liquid phase transition temperature does not fall below 41 °C (Christensen et al., 2008) which is important for *in vivo* applications if more rigid liposomes are required. The stabilising effect of TDB in DDA bilayers is attributed to the interaction of TDB glucose head-groups with surrounding water molecules of the aqueous hydrating solution (Christensen et al., 2008; Davidsen et al., 2005). It is therefore conceivable that changes to the hydrating solution, such as pH, molarity or buffer composition will have an effect on the physicochemical characteristics of the liposomal system. Whilst these properties have not been studied in great detail with DDA based liposomes, similar work using other cationic liposomes in which various concentrations of NaCl were added to the hydrating buffer showed significant alterations in both the physicochemical properties of the liposomes and in their ability to immunise mice in a tumour vaccine model (Yan and Huang, 2009). Improvements in immunogenicity were noted upon addition of small amounts of NaCl (30 mM) and the authors hypothesised that this improvement was due to altered physicochemical attributes such as an increase in vesicle size and zeta potential and facilitated antigen release *in vivo*.

3.2.3 Choice of route of administration for immunisation studies

Human vaccines are most commonly administered via the parental route with all protein based vaccines being given via intramuscular (i.m) injection whilst inactivated microorganisms are given predominantly via the subcutaneous (s.c) route. Although both the i.m and s.c routes of injection are preferential with regards to ease of administration, neither route is favoured amongst immunologists seeking to target immunologically privileged sites. For this, intradermal (i.d) administration is probably most favourable as the dermal layers of the skin comprise many Langerhans cells which are able to endocytose and present antigen (Kissenpfennig et al., 2005; Steinman and Banchereau, 2007). The i.d route is also becoming more popular due to its use in novel administration techniques such as transdermal skin patches and microneedles (Cevc and Vierl, 2010).

Consequently when delivery routes described in experimental studies published in more traditional vaccine journals are compared with those in journals focussing on delivery systems, the i.d route is preferential in the latter whilst i.m and s.c routes of administration are favoured in the former (Johansen et al., 2010). Figure 3.1 shows the tissue physiology encountered after i.m, i.d and s.c. injection.

Figure 3.1 Diagram showing simplified tissue physiology encountered after injection via intramuscular (i.m), intradermal (i.d) or subcutaneous (s.c) administration routes. Taken from (Chester, 1998).

3.3 General Methods

Liposomes composed of DDA, with or without TDB or cholesterol, were produced and their physicochemical characteristics identified. These DDA-based liposomes were used to investigate how model proteins ovalbumin (OVA) and bovine serum albumin (BSA) affect the aforementioned liposomes. The role of DDA-based liposomes in initiating an antigen depot-effect was investigated in biodistribution studies using dual-radiolabelled components; the effect of TDB and cholesterol are both documented. Furthermore, the role of 'administration route' in altering the depot-effect was analysed after injection via the i.m and s.c routes of injection.

To investigate whether vaccine delivery via different routes of administration had an effect on the ensuing immune responses, DDA and DDA:TDB liposomal vaccines delivered via i.m,

s.c, i.d or intralymphatic (i.ln) routes of injection were investigated in a collaborative study headed by Pål Johansen (University Hospital of Zurich, Switzerland). The principle aim of this study, also in partnership with Bruno Gander (ETH, Switzerland) and Wim Jiskoot (Leiden/Amsterdam Centre for Drug Research, Netherlands), was to investigate how non-adjuvanted and adjuvanted vesicular delivery systems delivered by various routes of injection effect the immune response of OVA immunised mice. In total three different delivery systems and four different routes of injection were studied with each delivery system being made in a different laboratory (Table 3.1 and 3.2). The i.ln injection route was to act primarily as a positive control as this has, unsurprisingly, been shown to improve the immune response to protein and peptide vaccines (Johansen et al., 2005; Martinez-Gomez et al., 2009; Senti et al., 2009).

Table 3.1 Outline of the various routes of injection used in collaborative studies with Pål Johansen, University Hospital of Zurich, Switzerland

Table 3.2 Formulations produced for collaborative work with Pål Johansen, University Hospital of Zurich, Switzerland

DDA, dimethyldioctadecylammonium bromide; TDB, trehalose 6,6'-dibehenate; TMC, N-trimethyl chitosan; LPS, lipopolysaccharide; PLGA, poly(lactide-co-glycolide).

The ability of DDA based liposomes to immunise mice and cause appropriate antibody and cytokine production was also investigated at Aston University, again using the model antigen OVA. The principle aim of this experiment was to investigate inter-researcher variation and to allow each researcher (MH, Malou Henriksen; RK, Randip Kaur; JH, Jubair Hussain) to gain experience of the experimental set-up in addition to gathering statistically significant data. Each step (formulation production, collection of blood, injections, spleen harvesting etc) of the study was conducted individually so to investigate inter-researcher variance. OVA was injected alone (non-adjuvanted), with Incomplete Freund's Adjuvant (ICF) (positive control) or with DDA:TDB liposomes (adjuvanted vaccine formulation).

Importantly, the choice of antigen (OVA), batch of antigen, adjuvant formulation (DDA:TDB liposomes), adjuvant dose (250 μg DDA, 50 μg TDB) and mouse strain (Balb/c) used in the immunisation experiment conducted at Aston University and at University Hospital of Zurich were the same and therefore allowed direct comparison between the results. The only variations between the two immunisation experiments involved the OVA dose, which was 10 μg/dose for studies conducted at Aston University and 20 μg /dose for those conducted at University Hospital of Zurich, and the researcher conducting the injections and analysis.

3.4 Results and Discussion

3.4.1 Physicochemical characterisation of DDA-based liposomes

Initial work focussed on the characterisation of the liposomal formulations and investigating how changes such as buffer molarity or addition of protein antigens have an effect on the physicochemical characteristics of the liposomes. The simplest liposome construct was composed of DDA alone, at a concentration of 1.25 mg lipid per ml of rehydrating buffer. Liposomes composed of DDA and rehydrated in Tris buffer (10 mM) were typically 403 ± 24 nm in diameter and expressed a polydispersity of 0.226 (Table 3.3). Polydispersity can be used as a measure of heterogenicity and differs from the standard deviation (SD) or standard error of the mean (SEM) as it assesses the whole time period which the sample is being measured for, whilst the SD or SEM is based on the individual values recorded by the machine. To clarify, if a liposome sample is assessed for 1 minute with 3 values recorded, the SD or SEM with be calculated from these 3 values. The PD

however will take into account the sample variance detected for the 1 minute period. PD can range from 0 (100 % homogenicity) to 1.0 (100 % heterogenicity). As no attempt was made during the liposome formulation to physically define the vesicle size range, the observed heterogenicity seen with DDA liposomes was to be expected and is considered quite low.

Table 3.3 Physicochemical characteristics of DDA and DDA:TDB liposomes

	DDA liposome (1.25 mg/ml), Tris buffer (10 mM)	**DDA:TDB liposome (1.25:0.25 mg/ml), Tris buffer (10 mM)**
Vesicle Size (nm)	403 ± 24	417 ± 33
Polydispersity	0.226	0.275
Zeta Potential (mV)	51 ± 12	45 ± 7

Results denote the mean ± SD of at least 3 experiments

Liposomes composed of solely DDA show instability over time (Davidsen et al., 2005), as measured by an increase in vesicle size due to particle aggregates forming. Although DDA liposomes are immunogenic, the addition of the synthetic bacterial cell wall glycolipid TDB has been shown to improve their immunogenicity and stability (Davidsen et al., 2005). Previous studies which investigated the effect of various molar ratios of DDA:TDB have shown that addition of 11 mol % TDB to DDA liposomes gave optimal immunogenicity and stability for the liposome formulation (Davidsen et al., 2005). Through the use of DSC, TDB was shown to incorporate into DDA bilayers with the carbohydrate head group in the hydrophilic partition of the bilayer (Davidsen et al., 2005). Liposomes composed of DDA and TDB (at the 11 mol % ratio, equivalent to a weight ratio of 1.25:0.25 mg DDA:TDB) had a vesicle size of approximately 417 ± 33 nm and a polydispersity of 0.275, neither of which were significantly different from DDA liposomes alone (Table 3.3). Importantly, the addition of 11 mol % TDB to DDA liposomes did not alter their zeta potential suggesting that the quantity of TDB added does not 'dilute out' the strong cationic charge attributed to the DDA head-group; DDA and DDA:TDB liposomes expressed zeta potentials of 51 ± 12 mV and 45 ± 7 mV respectively (Table 3.3). Vesicles produced from DDA and TDB were multilamellar, as shown using trace amounts of rhodamine labelled DPPE to identify the

bilayers (Figure 3.2). By conducting 'z-slices' through liposome samples mounted on a glass slide it was possible to see the 'onion-skin' appearance of DDA:TDB liposomes. The use of fluorescence and transmission election microscopy (TEM) highlights the heterogeneity of the vesicles produced, and the occasional non-spherical nature of the vesicles (Figure 3.3).

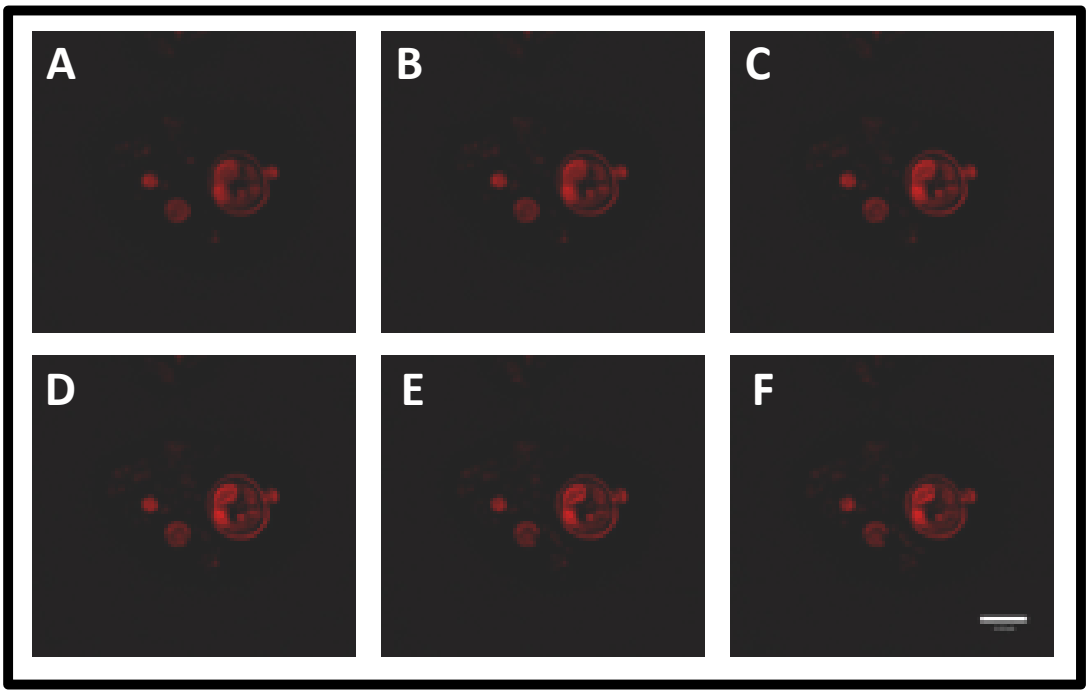

Figure 3.2 Z-stack series of DDA:TDB liposomes incorporating 1 mol % of Rho-DPPE lipid to act as a tracer stain of the bilayers. A->F, top to bottom of z-stack.

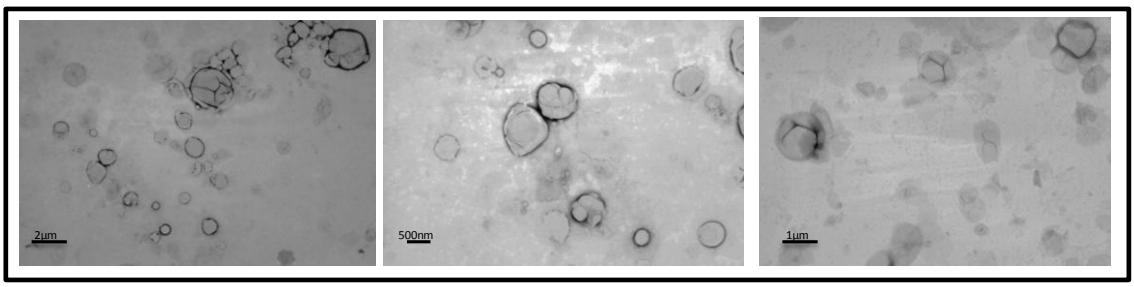

Figure 3.3 Transmission election microscope images of DDA:TDB liposomes.

The liposomes described above were all produced using Tris buffer (10 mM) to hydrate lipid films. One of the disadvantages of using Tris buffer is its non-isotonic nature; consequently an isotonic supplement must be added for any *in vivo* studies. A common

laboratory buffer which does not have this requirement is PBS; therefore we also investigated the effect of rehydrating the lipid-films with PBS buffer as opposed to Tris buffer. As noted in Figure 3.4, using PBS buffer of the same molarity as Tris buffer (10 mM) resulted in the production of significantly larger liposomes (~ 3000 nm). However, by reducing the molarity of PBS the vesicle size could also be reduced which suggests this change in size is a salt induced effect. No significant differences in the zeta potential were observed. TEM images also confirm the presence of larger DDA:TDB liposomes when produced using PBS buffer to hydrate lipid-films (Figure 3.5).

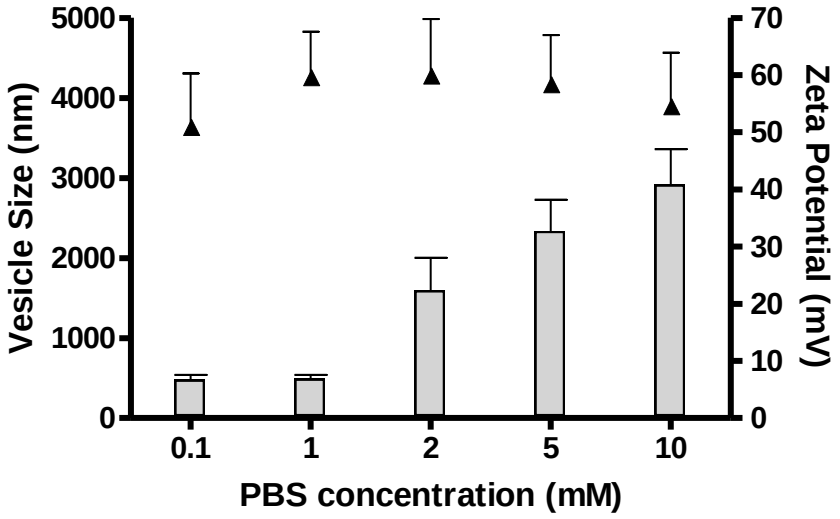

Figure 3.4 Vesicle size (bars) and zeta potential (points) of DDA:TDB liposomes rehydrated with varying molarities of PBS buffer. Samples were measured using a Brookhaven ZetaPlus and represent the mean ± SD of three separate readings.

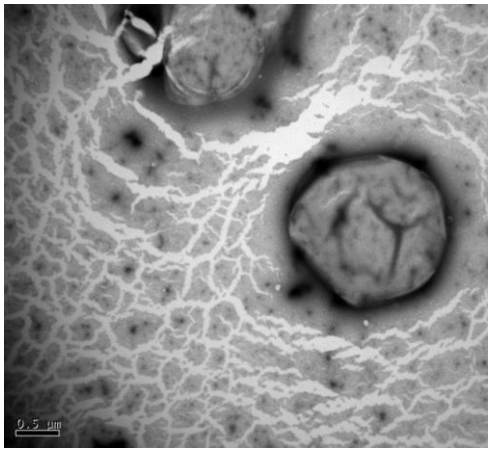

Figure 3.5 TEM image of DDA:TDB liposomes produced with PBS buffer (10 mM).

An important property for pharmaceutical preparations is stability. With this is mind, physicochemical properties of DDA:TDB liposomes were measured over time at room temperature (~ 20 °C) and also at 4 °C, thereby mimicking a cold-chain environment. Liposomes were produced using the standard lipid-film hydration method and a 1 ml aliquot placed in a glass vial so that visually any changes in the suspension could be observed. In addition, at certain time-points a sample of the liposomes was removed from a separate aliquot and their vesicle size, polydispersity and zeta potential measured. From Figure 3.6 it is clear that PBS buffer has a significant effect on the initial size of DDA:TDB liposomes and their stability over time. DDA:TDB liposomes produced with PBS buffer exhibit more variation in their mean vesicle diameter and zeta potential (Figure 3.6, C, D) whilst Tris buffer formed liposomes show no significant changes in the vesicle size over time. The physical instability of DDA:TDB liposomes formed with PBS buffer is seen visually in Figure 3.7 in which photos of 1 ml samples were taken at the same time-points. PBS buffer formed liposomes show clear phase separation just 1 day post formation whilst Tris buffer formed samples remain homogenous even after 56 days at room temperature.

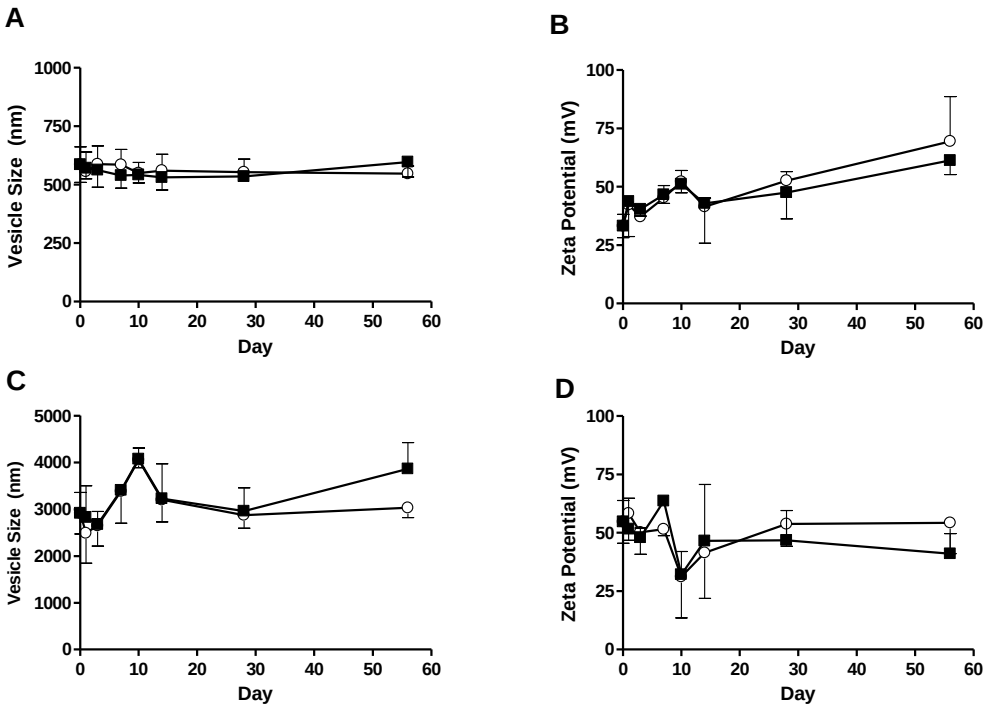

Figure 3.6 Variation on vesicle size (A,C) and zeta potential (B, D) of DDA:TDB liposomes made with a Tris (A,B) or PBS (C,D) buffer, both at 10 mM. Data is presented from samples stored at 4 °C (○) and room temperature (■). Samples were measured using a Brookhaven ZetaPlus and represent the mean ± SD of three separate readings.

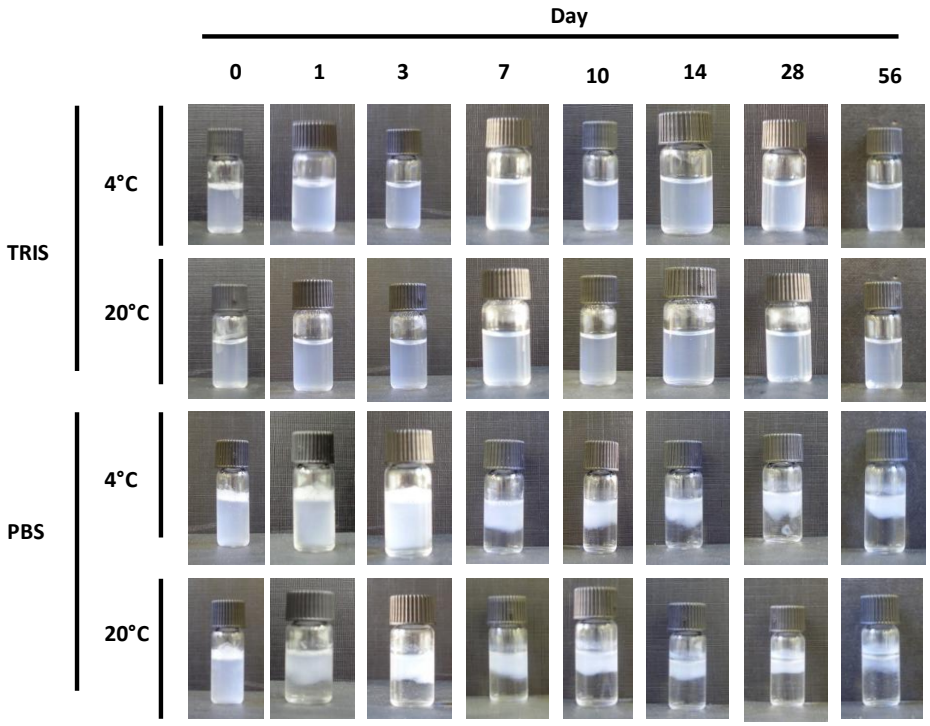

Figure 3.7 DDA:TDB liposome samples, made in either Tris or PBS buffer (both 10 mM) were stored for 56 days at 4 °C or 20 °C.

Salt-induced aggregation of charged vesicles has been reported previously but by simply reducing the molarity of the saline buffer, aggregation of vesicles can be prevented ((Kawakami et al., 2000; Ogris et al., 1998) and Figure 3.4). The presence of neutralising counterions in saline buffers has been suggested as the cause of aggregation, due to reduced repulsion between adjacent vesicles (Yan and Huang, 2009). Whilst the formation of aggregates such as those seen in Figure 3.7 is undesirable for pharmaceutical preparations requiring a long shelf-life, studies have shown that lipoplexes developed with these larger salt-induced aggregates induce 10 - 100 fold higher levels of transfection *in vitro* (Ogris et al., 1998). Furthermore, results derived from *in vivo* studies described improved transfection in lung (Kawakami et al., 2000) and improved immune responses for therapeutic cancer vaccines (Yan and Huang, 2009).

3.4.2 Addition of protein to DDA-based liposomes

DDA-based liposomes have a cationic surface charge (Table 3.3). Therefore, upon addition of molecules such as peptides or protein which have an isoelectric point (pI) of < 7 units (consequently expressing an overall anionic charge at neutral pH values), electrostatic-

mediated adsorption of the molecule to the liposome surface can occur. Two commonly used model proteins are ovalbumin (OVA) and bovine serum albumin (BSA), primarily used due to their availability, inexpensiveness and the large amount of data relating to them. OVA and BSA have a pI of 4.5 and 4.6 units respectively making them negatively charged at pH 7.4. The size of OVA and BSA proteins vary slightly with OVA containing 386 amino acids with an overall size of 45 kDa whilst BSA is composed of 607 amino acids and an overall size of 68 kDa. The effect of addition of different concentrations of OVA and BSA to DDA:TDB liposomes produced in Tris buffer was investigated primarily to see at what concentration liposomes become saturated with protein and the effect this has on the physicochemical properties. OVA and BSA were added to liposomes to produce a final concentration of 1, 5 of 10 mg protein per ml liposomes; liposomes were measured for their vesicle size, zeta potential and protein adsorption efficacy.

DDA:TDB liposomes made in Tris buffer originally expressed a mean vesicle diameter of ~ 600 nm and zeta potential of ~ 50 mV, however, upon addition of OVA or BSA there was an increase in vesicle size and decrease in zeta potential (Figure 3.8, A,B). Interestingly upon addition of OVA or BSA (1 mg/ml) there was an increase in vesicle size to ~ 3000 nm and a reduction to near neutral zeta potential. On further addition of either OVA or BSA, vesicle size decreased to ~ 2000 nm whilst the zeta potential continued to decrease to around -25 mV. Further increases in concentration of either protein resulted in no significant difference in vesicle size or zeta potential. These results suggest that addition of either protein results in aggregation of the liposomes into larger constructs which have their cationic charge neutralised by the anionic proteins. However the extent of aggregation is dependent on the liposome/protein ratio, or indeed liposome/nucleic acid ratio (McNeil et al., 2010; Ogris et al., 1998). This saturation effect is supported by the measurement of protein adsorption to the liposomes. The BCA assay was used to measure non-adsorbed protein in the supernatant of pelleted liposomes and not that associated with the liposome directly due to lipids interfering with the absorbance readout. Addition of either OVA or BSA to a final concentration of 1 mg/ml led to very high levels of adsorption (~ 0.8 to 0.9 mg/ml; Figure 3.8, C). Further addition of either protein did not greatly increase the proportion of protein adsorbed to the liposomes (e.g. ~ 30 % of 5 mg/ml was determined to be adsorbed to the liposomes which is equivalent to 1.5 mg/ml, and addition of 10 mg/ml OVA or BSA resulted in ~ 15 % adsorption equivalent to ~ 1.5 mg/ml; Fig 3.8 C), suggesting a saturation point had been reached.

Similar high levels of OVA adsorption to DDA:TDB liposomes, made in Tris buffer, were also detected using radiolabelled OVA validating the above BCA method. In this case the concentration of OVA added to liposomes was less as the formulations were intended for *in vivo* immunisation studies conducted at University Hospital of Zurich. The final OVA concentration was 100 μg/ml liposomes and of this, 88.8 ± 0.33 % of OVA was found adsorbed to the liposomes (Figure 3.8). Measurement of OVA association after 18 hrs at 37 °C showed high retention with 74.79 ± 1.39 % of OVA still adsorbed to DDA:TDB liposomes (results not shown). In a further immunisation study conducted here at Aston University, the effect of OVA (final concentration 50 μg/ml) on DDA:TDB liposome made in Tris buffer was investigated by measuring the vesicle size and zeta potential of liposomes. Upon addition of OVA to a final concentration of 50 μg/ml, no significant changes in vesicle size or zeta potential are noted, presumably due to the low protein concentrations employed (Figure 3.8, A, B).

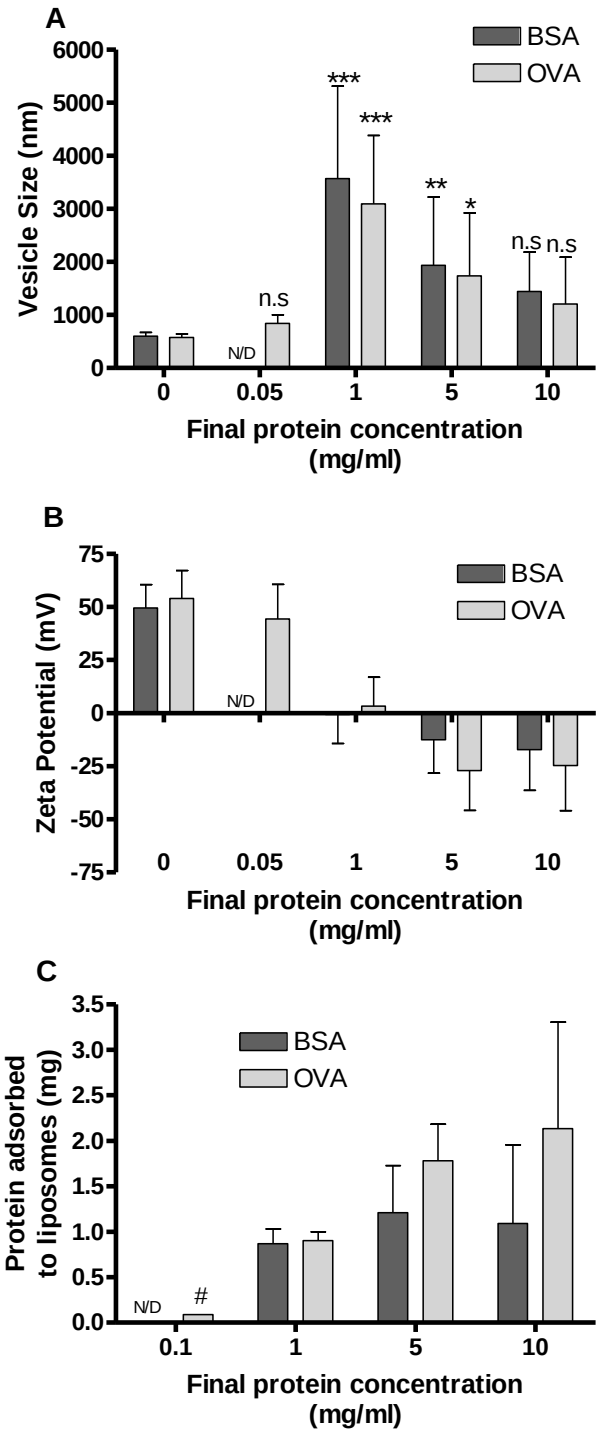

Figure 3.8 The effect of addition of OVA or BSA to DDA:TDB liposomes made with Tris buffer. Proteins were added to liposomes at a final concentration of 0.05, 0.1, 1, 5 or 10 mg protein/ml liposomes. Vesicle size (A) and zeta potential (B) were measured using a Brookhaven ZetaPlus machine. Protein adsorption was determined using the BCA assay (or radiolabelling, #) and is expressed as a % of protein added. Where shown, significance between data is against results of 0 mg/ml protein and was calculated using 2-way ANOVA from the mean values of 4 separate studies (n.s, not significant; *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$). N/D, not determined.

Due to the wide OVA concentration range that can be produced, OVA is an ideal protein to study protein-induced changes on liposomal systems. However, OVA is not a disease model antigen and whilst it is commonly used in immunisation studies, vaccination with OVA as an antigen does not protect against disease. We therefore moved towards the use of the subunit protein Ag85B-ESAT-6, also known as H1, developed as an antigen for immunisation against tuberculosis (Olsen et al., 2001). Ag85B-ESAT-6 is approximately 44 kDa and has a pI of 4.9 units making it similar in size and pI to OVA. The pI of Ag85B-ESAT-6 favours its association via electrostatic interactions with cationic charged particles such as DDA:TDB liposomes. For *in vivo* studies, each liposome dose (250 µg lipid) contained 2 µg Ag85B-ESAT-6. This is equivalent to an antigen concentration of 10 µg/ml for liposomes made to the usual 1.25 mg lipid/ml concentration. Ag85B-ESAT-6 was supplied from Statens Serum Institute at a concentration of 0.8 mg/ml and therefore was generally used at low concentrations (bearing in mind the relevant dilution factors upon addition to liposomes). Hence, due to the concentration constraints of Ag85B-ESAT-6, much of the initial characterisation work described previously was conducted with OVA with which higher concentrations could be obtained.

Figure 3.9 shows the effect of adding Ag85B-ESAT-6 (final concentration 10 µg/ml) to both DDA and DDA:TDB liposomes. No significant changes in size were observed upon addition of the TDB glycolipid or the Ag85B-ESAT-6 antigen due to the low concentrations of both components used. TDB was incorporated into DDA liposomes to a final concentration of 11 mol %, based on previous results documenting improved physicochemical and immunological results compared to lower (6 mol %) or higher (20 mol %) TDB concentrations (Davidsen et al., 2005).

As mentioned previously, the use of Ag85B-ESAT-6 as opposed to OVA was due to the requirement of an antigenic protein for immunisation studies. It was therefore of interest to observe the effect of exposure of the liposomal system to an *in vivo* environment. Liposomes adsorbing Ag85B-ESAT-6 were consequently exposed to a 50 % v/v solution of foetal calf serum (FCS) with Tris buffer at 37 °C. As seen in Figure 3.9 immediate liposome aggregation is observed and the surface charge of liposomes decreases to approximately -17 mV due to the adsorption of anionic serum proteins, also noted with other cationic liposomal systems (Oku et al., 1996; Zelphati et al., 1998). Continued exposure to FCS for a period of 96 hrs did not change the observed aggregation or decrease in zeta potential: by 96 hrs vesicle sizes of ~ 2 µm and a zeta potential of -22 mV were observed for both DDA

and DDA:TDB liposomes (results not shown). Ag85B-ESAT-6 adsorbed to DDA and DDA:TDB liposomes to a very high degree and was well retained in conditions simulating the *in vivo* environment (Figure 3.10). Over the 96 hr measurement period no more than 13 % of the original amount of Ag85B-ESAT-6 added to the liposomes was found to be released from the liposomes; constraints with sterility of the FCS by this time meant that the experiment could not be continued. However these results show the strong adsorption abilities of DDA based liposomes and when added at concentrations of < ~ 50 µg/ml, no protein induced physicochemical changes to the DDA-based liposomes are observed.

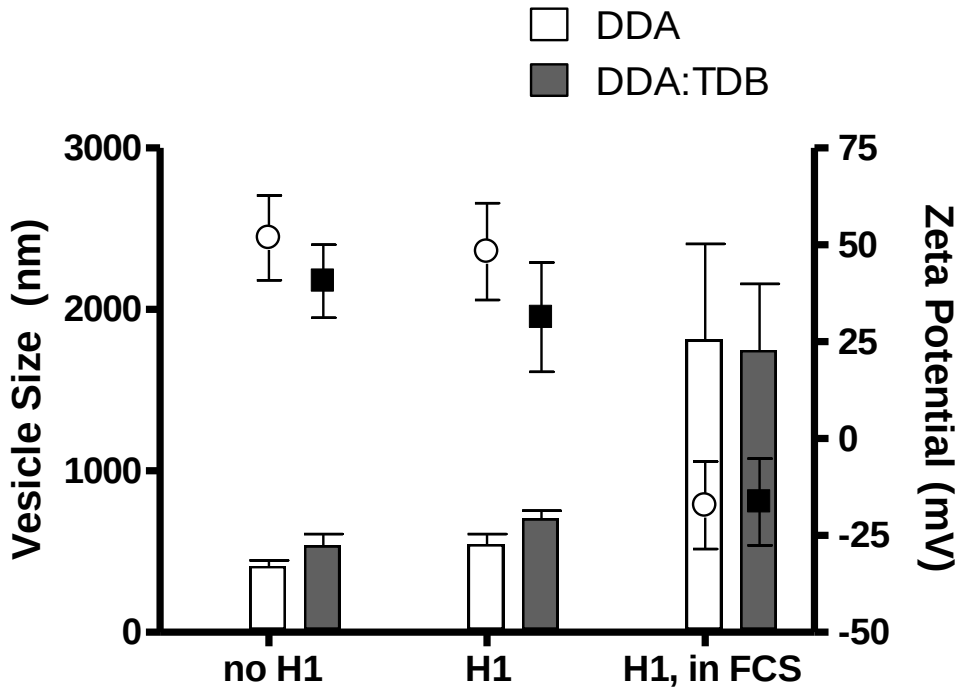

Figure 3.9 The effect on the vesicle size (bars) and zeta potential (points) of DDA or DDA:TDB liposomes after addition of Ag85B-ESAT-6 (H1) and subsequent placement in 50 % FCS at 37 °C for 1 hr. Results denote mean ± SD of three separate experiments in which the pellet obtained after 2 washes was measured.

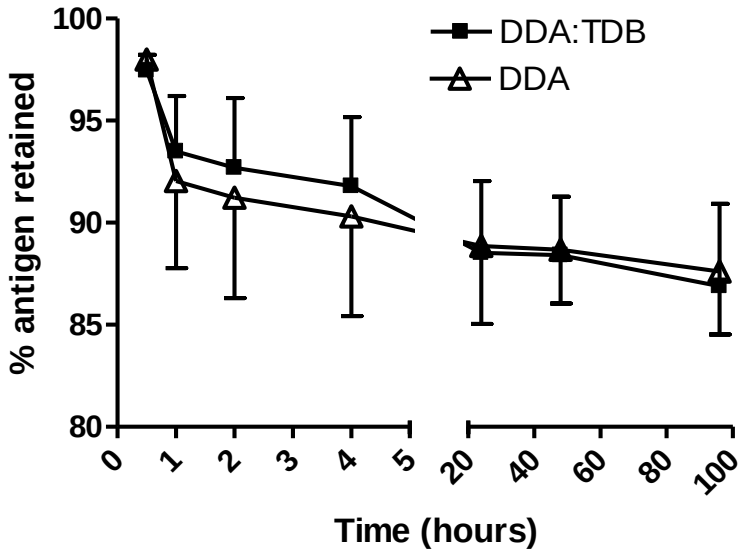

Figure 3.10 Ag85B-ESAT-6 antigen retention profile to DDA and DDA:TDB liposomes when stored at 37 °C in 50 % FCS. Results denote mean ± SD of three separate experiments.

3.4.3 Membrane stability of DDA-based liposomes

DDA liposomes are unstable structures but the addition of the immunomodulatory molecule TDB is able to stabilise the vesicles and inhibit aggregation (Davidsen et al., 2005). Although liposome bilayers can be considered as a fluid system with movement between lipid monomers, loss of lipid components into the surrounding aqueous media does not occur because of the hydrophobic nature of the hydrocarbon chains and the critical micellar concentration (CMC) of the lipid. A simple method to test membrane stability is the incorporation of a trace amount of radiolabelled lipid whose presence either in the liposome or 'leaked' into surrounding media can be measured. To conduct this study, ^{3}H-labelled DPPC was employed – ^{3}H-DPPC was also used to detect liposome presence *in vivo* in biodistribution studies (this will be discussed in section 3.4.6). As only tracer amounts were used, no physicochemical changes in the system were noted (Table 3.4). Dialysis was used to study the retention of ^{3}H-DPPC in the liposome membrane; aliquots of dialysis media were removed at various time-points and the amount of ^{3}H present measured. Figure 3.11 shows the time scale of ^{3}H-DPPC release from the DDA:TDB liposomal membrane. Over the 96 hour period ~ 7 % of the original ^{3}H-DPPC dose was recovered supporting the hypothesis that lipid membranes remain intact under *in vivo* conditions.

Table 3.4 Physicochemical characteristics of DDA:TDB liposomes with DPPC lipid

	DDA:TDB liposome (1.25:0.25 mg/ml)	DDA:TDB liposome (1.25:0.25 mg/ml), incorporating 0.17 nM DPPC
Vesicle Size (nm)	578 ± 61	589 ± 72
Polydispersity	0.256	0.301
Zeta Potential (mV)	54 ± 13	48 ± 9

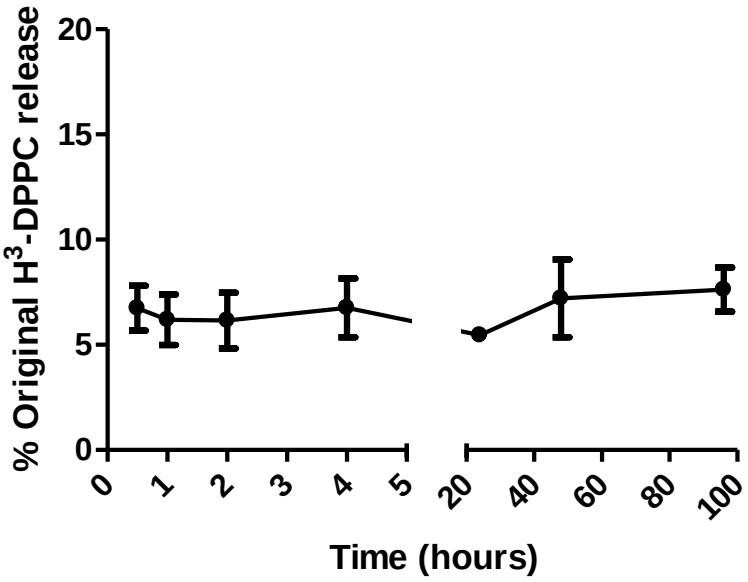

Figure 3.11 Membrane integrity of DDA:TDB liposomes was studied using a trace amount of ^{3}H-DPPC. Results express the % of the original ^{3}H-DPPC dose added to liposomes that is detected in the dialysis buffer over a 96 hr time period. The samples were stored at 37 °C in 50 % FCS. Results denote mean ± SD of three separate experiments.

3.4.4 Attempts to improve salt-induced aggregation with the stabilising component cholesterol

A further commonly used method to stabilise liposomes is the addition of cholesterol. Cholesterol is a natural component of mammalian cell membranes and provides rigidity due its large structure acting as a space filling component. Cholesterol was included in DDA:TDB liposomes at a molar ratio of 8:4:1 DDA:CHOL:TDB and both Tris and PBS buffers were used for the formation of the liposomes so that the stabilising effects of cholesterol could be investigated in the otherwise highly unstable PBS buffer formed liposomes

(Figure 3.6, C). As seen in Figure 3.12, the addition of cholesterol to DDA:TDB liposomes was not able to stabilise PBS buffer formed liposomes as phase-separation is noted regardless of the temperature at which liposomes are stored. Cholesterol containing Tris-buffer formed liposomes were slightly larger than their non-cholesterol containing counterparts, however, their zeta potential did not differ significantly (Table 3.5). The stability of these cholesterol-containing formulations over time at 4 °C and at room temperature was investigated and whilst Tris buffer formed liposomes showed stability over the 56 day period, the size of PBS buffer formed liposomes varied significantly (Figure 3.13, bars). Whilst cholesterol has been shown to improve bilayer stability by limiting plasma protein association (Gregoriadis and Davis, 1979; Semple et al., 1996), it does not appear to be able to improve bilayer stability of liposomes which show aggregation in buffers with a high salt content such as PBS.

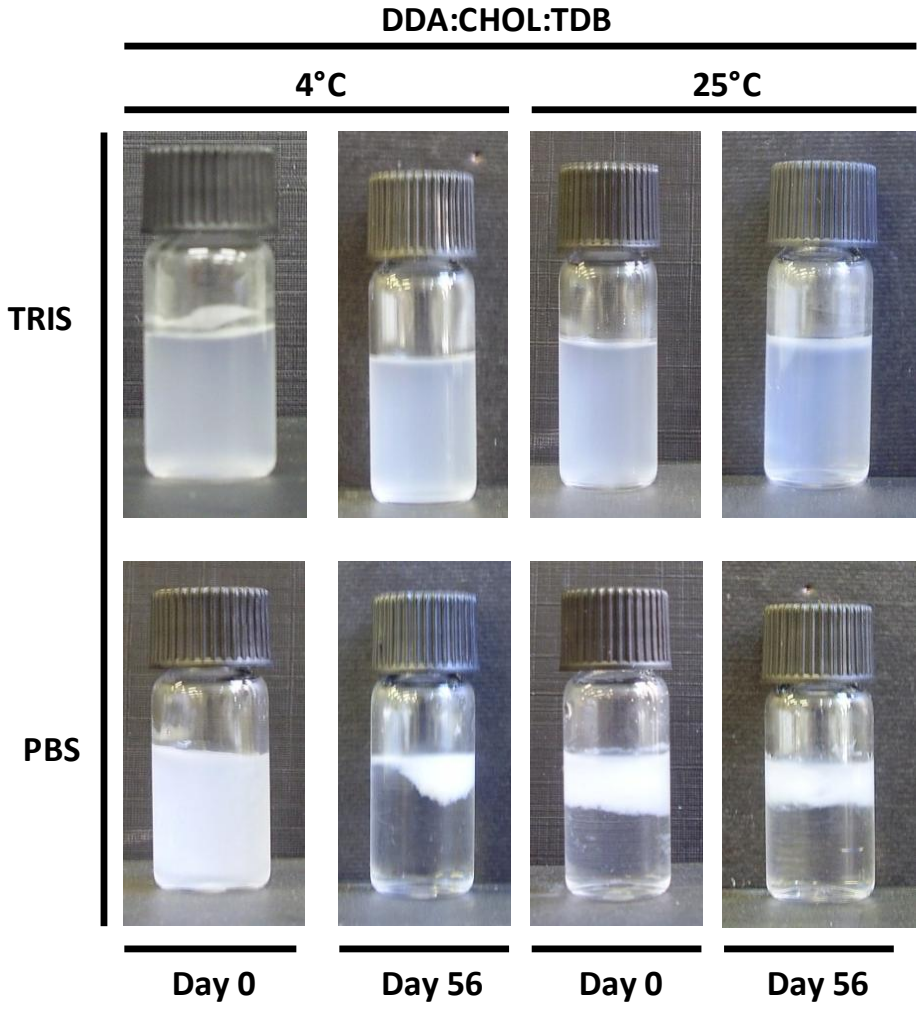

Figure 3.12 PBS buffer induced DDA:TDB liposome instability ensues regardless of whether cholesterol is or is not included in the formulation.

Table 3.5 Physicochemical characteristics of DDA and DDA:TDB liposomes.

	Vesicle Size (nm)	Zeta Potential (mV)	Ag85B-ESAT-6 adsorption (%)
DDA:TDB – Tris buffer	586 ± 78	33 ± 5	97.5 ± 0.8
DDA:CHOL:TDB - Tris buffer	737 ± 53	42 ± 3	97.2 ± 0.2
DDA:TDB – PBS buffer	2916 ± 446	55 ± 9	n.d
DDA:CHOL:TDB – PBS buffer	2573 ± 632	56 ± 10	n.d

n.d; not determined

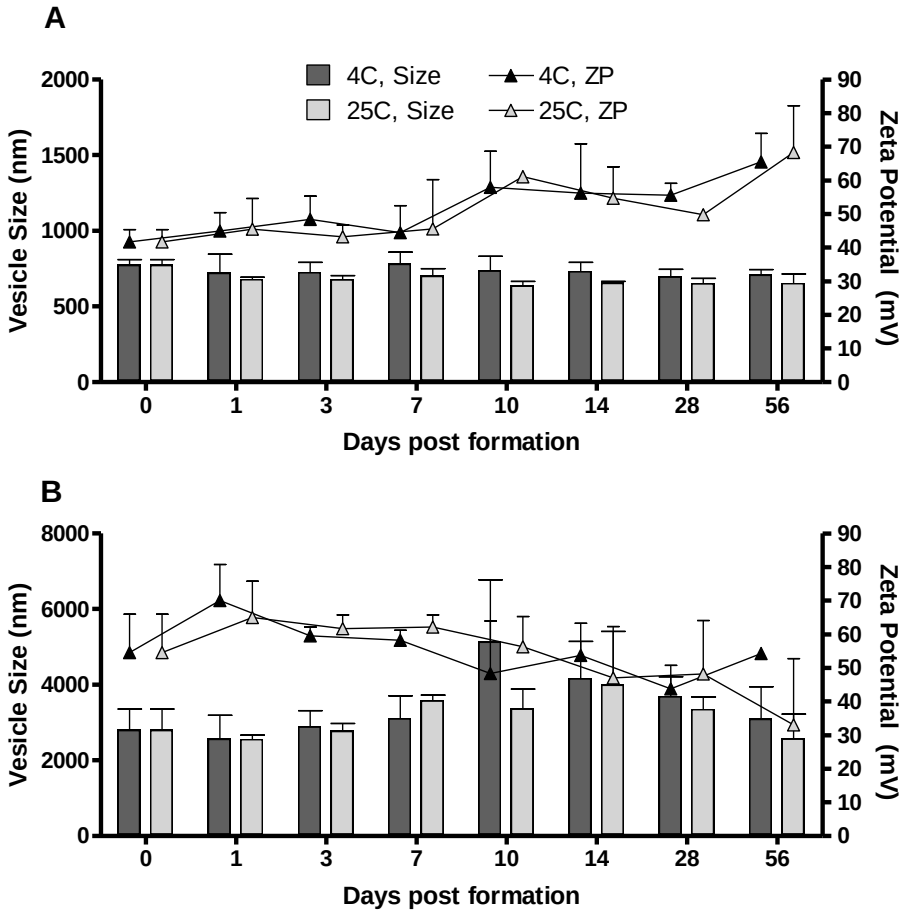

Figure 3.13 DDA:CHOL:TDB liposome vesicle size (bars) and zeta potential (points) was measured over a 56 day period to investigate the stability of the formulations. DDA:CHOL:TDB liposomes were produced using Tris (A) and PBS (B) buffers, both 10 mM. Results represent mean ± SD of three individual samples stored at 4 °C (▲) or room temperature (Δ) as depicted.

3.4.5 *In vitro* toxicity testing of DDA-based liposomes

Cationic liposomes are well known for their toxicity in comparison to neutral or anionic liposomes (Filion and Phillips, 1997). This is partly due to affinity to cell surfaces encouraging liposomal uptake and lysis upon excessive endocytosis. A simple method to test their toxicity is to apply various concentrations of liposomes to cell monolayers and then measure the cell viability after a certain period of time. The monocyte/macrophage cell line RAW264 was used to study the lipid-mediated toxic effects of liposomes. Initially the MTS assay was used, however, large variance was noted and therefore the neutral red (NR) assay was used instead in conjunction with the LDH assay. The NR and LDH assays complement each other because the NR assay measures internalised NR dye in viable cells whilst the LDH assay measures leaked LDH enzyme that is present in the extracellular media due to lipid-associated cell membrane damage. The lipid-mediated toxic effects of DDA based liposomes were measured over a range of lipid concentrations applied for a period of 24 hrs. As shown in Figure 3.14, the highest lipid concentration that did not result in a significant decrease in cell numbers was 5 µg/ml which is in agreement with findings by Korsholm et al. in which lipid mediated toxicity on murine bone-marrow dendritic cells was evaluated (Korsholm et al., 2006). At the highest concentration (50 µg/ml) DDA:TDB liposomes were significantly ($p<0.001$) more toxic than DDA:CHOL:TDB liposomes (Fig. 3.14 A,B).

The results obtained from the NR and LDH assays are in strong agreement with each other and show that between the lipid concentrations 0.005–5 µg/ml the liposomes do not mediate toxic effects characterised by decreases in cell viability. Of note is that the LDH specific lysis results were calculated from the positive control in which cells were lysed with Triton® X-100. This is the recommended lysing solution by Promega and therefore this assumes that Triton® X-100 does indeed fully lyse cells. Whilst the toxicity of cationic liposomes has been attributed to factors such as reversal of the cell surface charge (Campanhã et al., 1999; Carmona-Ribeiro et al., 2006), the toxicity noted here is most probably due to excessive phagocytosis by the RAW264 cells initiated by electrostatic interactions between the anionic cell surface and the cationic liposomes (Filion and Phillips, 1997). Filion et al. also noted increased toxicity upon incorporation of fuseogenic lipids and decreased toxicity when liposomes were applied to cells with decreased phagocytic abilities (Filion and Phillips, 1997).

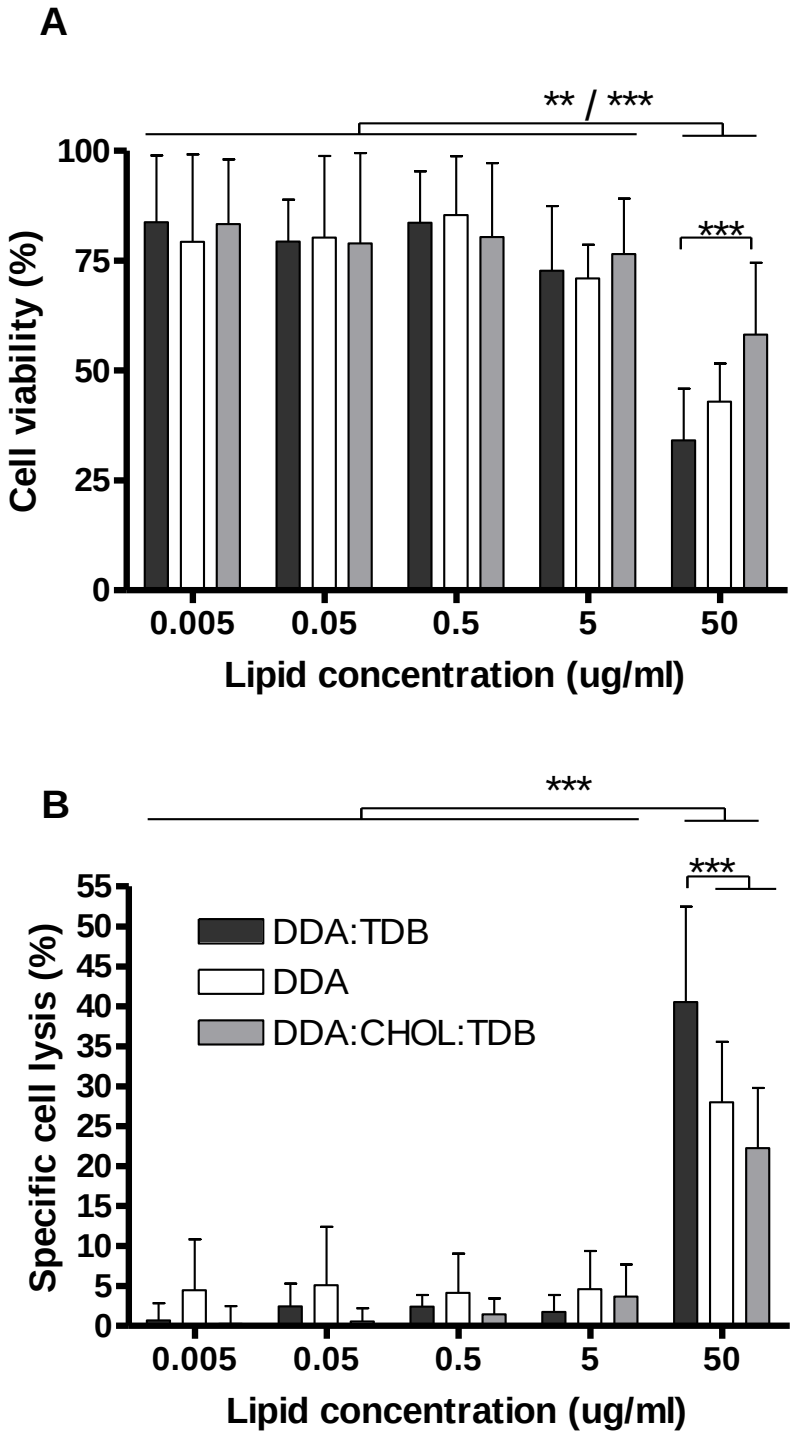

Figure 3.14 Cell viability measured using the neutral red (A) and LDH (B) assays to determine the highest lipid concentration that could be applied to RAW264 cells without significant loss of cell numbers. Results indicate the mean ± SD of three separate experiments in which four wells of a 96-well microplate were assayed per condition. A; cell viability was calculated as a % of cells without any liposomes therefore considered 100 % viable. B; Specific cell lysis is calculated as a % of maximum cell lysis as obtained by Triton® X-100. Lipid concentrations refer to the final DDA concentration/ml. Two way ANOVA was used to determine significance levels (**, $p<0.01$; ***, $p< 0.001$).

The MTS assay was originally used to measure cell viability and whilst it did give the same mean cell viability as the NR assay (MTS, 74.8 ± 24.8 %; NR, 72.7 ± 14.7 %), the well to well and inter-experiment variance was significantly greater. There are two principle reasons why a larger variance was seen upon implementation of the MTS assay as opposed to the NR assay. Firstly the MTS reagent was viscous and therefore small differences in the volume of MTS reagent applied per well may have lead to inaccuracies. Secondly the MTS assay relies on active mitochondrial metabolism for the successful reduction of the MTS reagent to a coloured product. In comparison the NR assay relies on lysosome activity (as the NR dye must be uptaken and blocked in lysosomes) and intact cell membranes. Therefore it would be expected that the MTS assay could be more prone to variation due to factors such as cell passage, the stage of their cell cycle and their 'healthiness'. Taken together, for the purposes of determining liposome-mediated toxicity in future experiments, the NR assay was implemented and not the MTS assay. Furthermore, the NR assay was chosen preferentially over the LDH assay because of its ease of use and inexpensiveness compared to the LDH assay.

3.4.6 Biodistribution studies: the liposomal depot-effect

DDA-based liposomes have previously been described as causing a depot-effect at the site of injection (SOI). However, no studies have quantified the retention of either the liposomal or antigenic component. Using a dual-radiolabelling technique, the biodistribution of liposomes and antigen were studied in mice. Tissues, including the spleen, liver, lung and importantly the lymph nodes which drain tissue antigen from the SOI, were removed and tested for the relative proportions of DDA-based liposomes and simultaneously injected Ag85B-ESAT-6 antigen (Figure 3.15). Regardless of the time-point post injection (p.i), the proportion of liposome detected in the various tissues was higher than the proportion of antigen detected (Fig. 3.15 A vs B). Also of note is the trend of increasing liposome presence in most tissues over the 14 day period whilst antigen presence generally decreases over time. In general, the level of DDA:TDB liposomes or Ag85B-ESAT-6 was low (< 0.5 % of the injected liposome dose) for all tissues except the site of injection (SOI) and therefore only the muscle from the SOI and the local draining lymph nodes were investigated further.

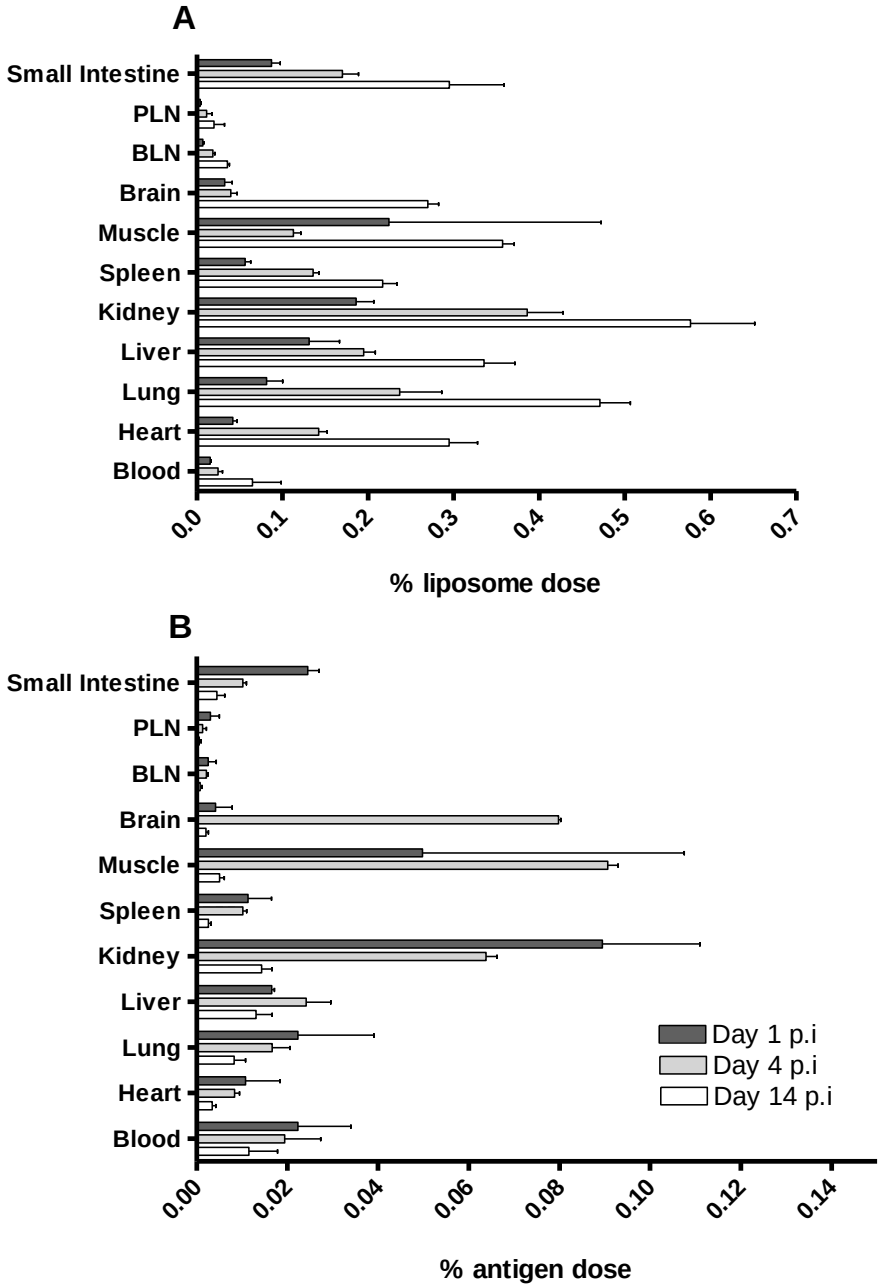

Figure 3.15 Detection of DDA:TDB liposomes (A) and adsorbed Ag85B-ESAT-6 antigen (B) in various tissues. Muscle from the site of injection has been omitted for clarity as the proportion of antigen and liposome dose are much higher. Instead, muscle from the non-injected leg is shown. Results represent mean ± SD of three mice. PLN, popliteal lymph node; BLN, brachial lymph node; p.i, post injection.

The role of the immunomodulator TDB and the stabilising component cholesterol in eliciting a liposome or antigen depot-effect was investigated. DDA, DDA:TDB, and DDA:CHOL:TDB liposomes adsorbing Ag85B-ESAT-6 were administered to Balb/c mice via

the i.m route of injection, and tissues removed on days 1, 4 and 14 p.i to analyse the proportion of liposome and antigen present. Using radiolabelled antigen, the level of Ag85B-ESAT-6 adsorption to DDA:CHOL:TDB liposomes was investigated and was shown to be as high (97.2 ± 0.2 %) as that seen for DDA and DDA:TDB liposomes with less than 10 % loss over 96 hrs when stored in 50 % FCS at 37 °C (results not shown). Figure 3.16 shows the presence of liposome (A) and Ag85B-ESAT-6 antigen (B) at the SOI after i.m injection of antigen adsorbing formulations. DDA, DDA:TDB and DDA:CHOL:TDB liposomes were all highly retained at the SOI with between 40 - 50 % of the original dose being recovered 2 weeks p.i (Figure 3.16, A). All three formulations showed a trend of decreasing liposome presence at the SOI with time although a significant decrease compared to day 1 p.i was only noted for DDA liposomes by day 14 p.i ($p<0.01$). This may be due to the stabilising effects of TDB and/or cholesterol which are known to improve liposome bilayer stability (Davidsen et al., 2005; Gregoriadis and Davis, 1979).

Ag85B-ESAT-6 injected without a liposomal carrier was cleared quickly with < 6 % of the injected dose detected on day 1 p.i and less than 0.5 % by day 14 p.i. In contrast, the retention of Ag85B-ESAT-6 at the SOI when injected with a liposomal carrier was very high on day 1 p.i with between 75 - 77 % of the original dose being retained at the SOI (Figure 3.16, B). Similar levels of Ag85B-ESAT-6 were detected on 4 p.i regardless of whether antigen was injected with DDA, DDA:TDB or DDA:CHOL:TDB liposomes. However, by day 14 p.i a significant decrease in Ag85B-ESAT-6 at the SOI was only noted after delivery with DDA liposomes. This suggests an important role for simultaneous liposome retention at the SOI for an increased antigen depot-effect (Figure 3.16, A and B). Importantly, antigen could still be detected at the SOI 2 weeks p.i with between 1 - 7 % of the original dose being recovered, regardless of the liposome used for delivery. The importance of the antigen depot-effect remains unclear, however it is accepted that antigen retained at sites with increased exposure to immune cells will lead to increased uptake and subsequent presentation on APCs (Obst et al., 2007).

With regards to movement of vaccine components to the local draining popliteal lymph node (PLN), DDA:TDB presence was significantly higher ($p<0.01$) at day 4 p.i and by day 14 p.i liposome presence was approximately 5-fold higher than on day 1 p.i ($p<0.001$) (Figure 3.17). Furthermore, the addition of the immunomodulatory molecule TDB resulted in an increased draining of DDA-based liposomes to the PLN ($p<0.05$ by day 14 p.i). The

presence of Ag85B-ESAT-6 in the PLN was low at all time-points and difficult to detect accurately (Figure 3.15, B).

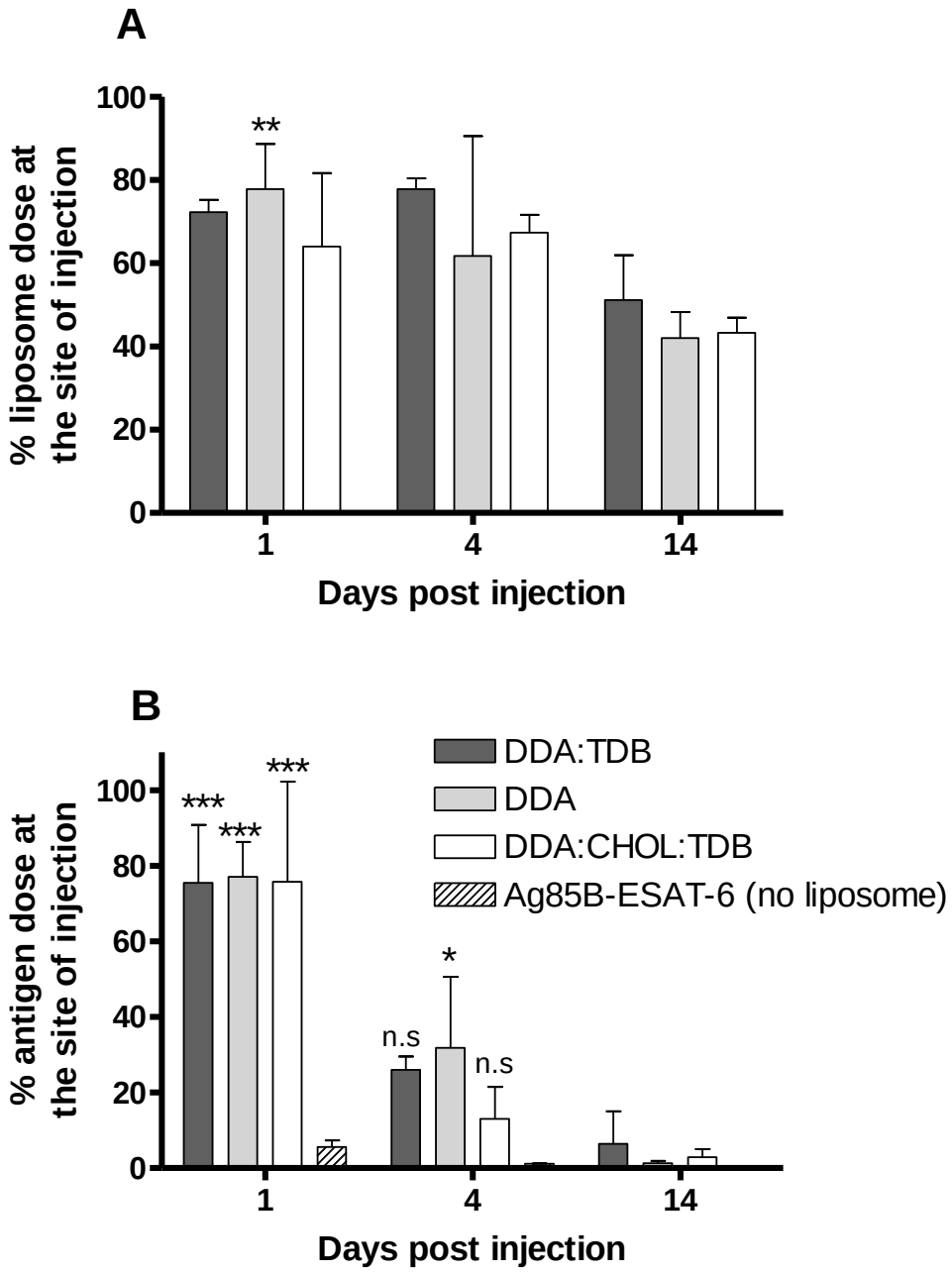

Figure 3.16 Presence of liposome (A) and antigen (B) at the site of injection following intramuscular injection of Ag85B-ESAT-6 antigen adsorbing to DDA-based liposomes. Tissue was collected on days 1, 4 and 14 p.i and assayed for the presence of ^{3}H and ^{125}I relating to liposome and antigen respectively. Results represent mean ± SD of three mice. Significance is against results from day 14 of the same formulation and was determined using 2-way ANOVA (*, $p<0.05$).

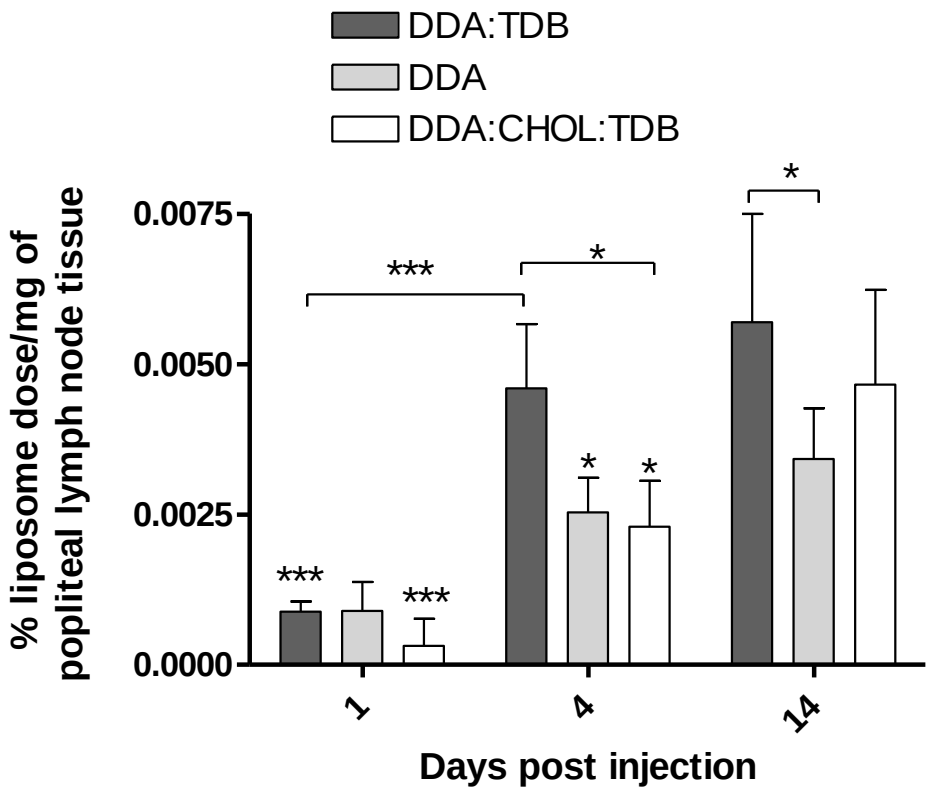

Figure 3.17 Liposome recovery from the draining popliteal lymph node (PLN) shown as a % of the original dose divided by the mass (mg) of PLN. Results show mean ± SD of three mice. Significant differences were measured using 2-way ANOVA (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$) and unless bracketed, significance is against day 14 p.i of the same formulation.

I.m injection leads to a strong retention of antigen and especially liposomes in the muscle (Figure 3.16). The quadriceps muscle is the largest muscle that can be injected into and whilst the injection volume (50 µl) is kept as low as is possible (with respect to liposome formulation), it is possible and likely that the muscle cannot retain such a volume. Furthermore the tissue physiology of muscle does not allow for massive dispersion of injected materials when compared to intravenous or intraperitoneal injection for example. Another commonly used route of injection is the subcutaneous (s.c) route due to the ease of administering substances with a larger volume. For this reason the s.c. route was also studied to see whether an antigen depot-effect could also be initiated upon injection with DDA:TDB liposomes. Figure 3.18 outlines the pharmacokinetics of DDA:TDB and associated Ag85B-ESAT-6 at the SOI (A) and in the brachial lymph node (BLN; B) which is the local draining lymph node to the injection site. Unfortunately the entire dose was not collected

from the SOI on day 1 p.i and therefore no data is presented. However by day 4 and 14 p.i the entire tissue was successfully collected and the liposome presence shows a similar trend to DDA:TDB liposomes following i.m injection. Also presented is the movement of freely administered Ag85B-ESAT-6 antigen, without the use of a liposome carrier. Compared to Ag85B-ESAT-6 administration with DDA:TDB liposomes, freely administered antigen drains rapidly and the proportion of antigen remaining in the subcutaneous tissue is minute (Fig. 3.18, A). In agreement with i.m injection, liposome presence in the local draining lymph nodes increases steadily over time and shows the same increase in the levels of significance over the 14 day period (Fig. 3.18, B).

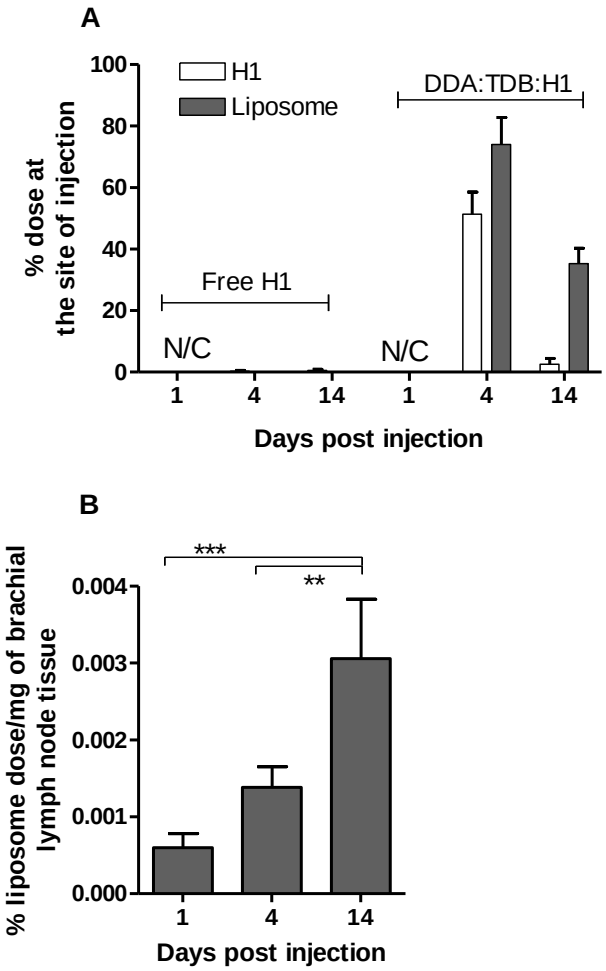

Figure 3.18 A; Presence of freely administered Ag85B-ESAT-6 antigen and Ag85B-ESAT-6 adsorbing to DDA:TDB liposomes at the site of injection after subcutaneous injection into the flank. B; DDA:TDB liposome presence in the local draining brachial lymph nodes – Ag85B-ESAT-6 antigen presence is not shown due to the low values obtained. Results represent mean ± SD of three mice. Significant differences were measured using 2way ANOVA (**, $p<0.01$; ***, $p<0.001$). N/C; not collected due to difficulties isolating the entire injected dose from the subcutaneous tissue. H1, Ag85B-ESAT-6. Experiment conducted in collaboration with Vincent W Bramwell.

3.4.7 Pontamine blue dye and its use as a leukocyte marker

Pontamine blue is an azo dye that has been described as being uptaken by macrophages *in vivo* therefore allowing for the identification of lymphoid tissue such as lymph nodes (Tilney, 1971). Although pontamine blue was primarily employed as a lymph node identification marker, it also served as a marker for identification of infiltrating macrophages to the SOI. Studies *in vitro* using the monocyte/macrophage cell line J774 confirmed the ability of the cells to take up the dye (Figure 3.19). Furthermore, pooled blood from mice injected with pontamine blue (0.5 % w/v) 3 - 5 days prior to vaccine injection also showed the presence of pontamine blue within cells (Figure 3.20). Cytospins of red-blood cell lysed blood were mounted with a hard-mount containing DAPI to stain nuclei. Although the specific cells taking up pontamine blue were not identified, based on the nuclei morphology there appears to be pontamine blue uptake by neutrophils (open arrows) and monocyte-like cells (closed arrows) (Figure 3.20, B).

Figure 3.21 shows the pontamine blue staining seen at the SOI 14 days after i.m injection. The intensity of blue staining was strongest for TDB containing DDA liposomes and weakest after injection of Ag85B-ESAT-6 antigen alone. Interesting the blue staining was more intense for DDA liposomes containing TDB as opposed to solely DDA, although the proportion of liposomes found at the SOI were not significantly different for either formulation (Fig. 3.16, A). Inclusion of cholesterol in the DDA:TDB liposomes did not have any effect on the intensity of pontamine blue staining noted at the injection site (Figure 3.22). Importantly, DDA liposomes incorporating cholesterol also contained the strong immunomodulator TDB which is proposed to play a more significant role on macrophage influx. Therefore the similar blue staining seen between DDA:TDB and DDA:CHOL:TDB formulations was to be expected.

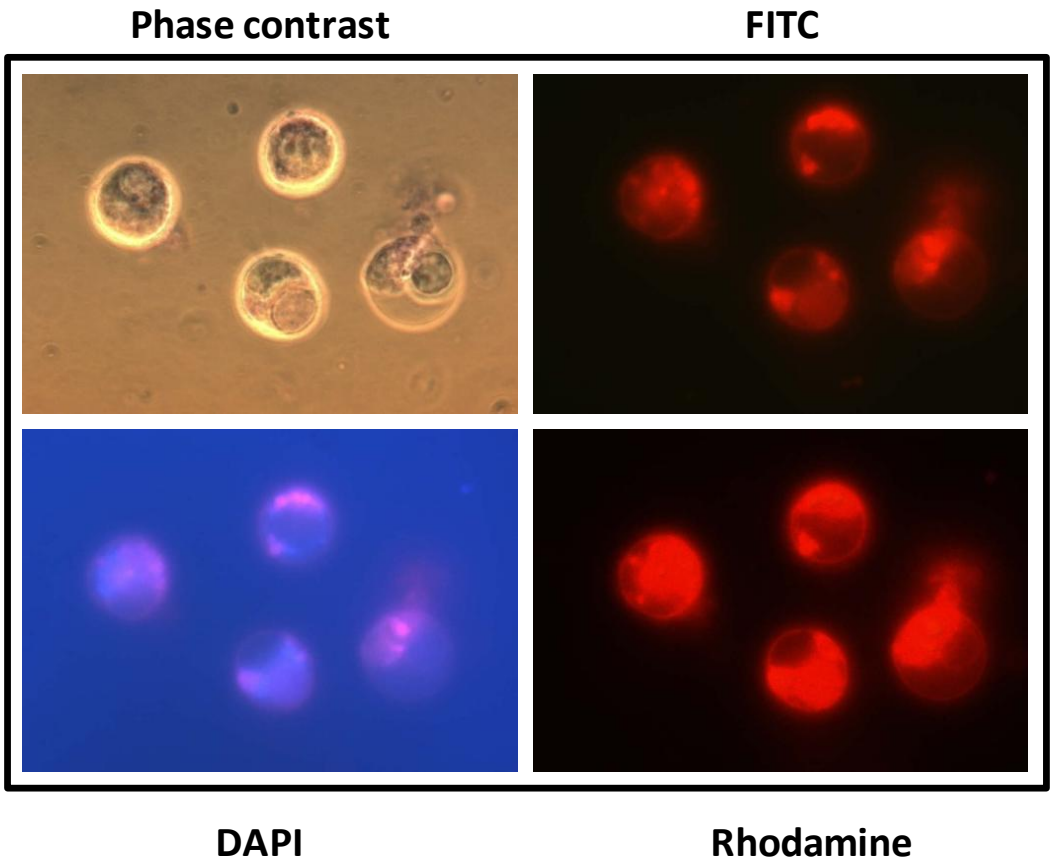

Figure 3.19 Pontamine blue (0.05 %) added to J774 cells is uptaken and can be viewed under FITC, DAPI or Rhodamine filters.

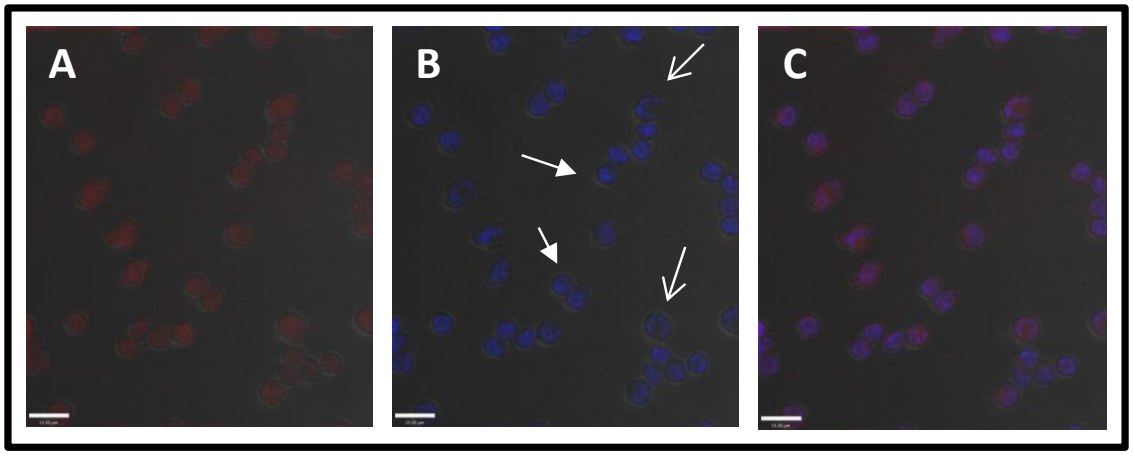

Figure 3.20 Cytospins of pooled blood from mice subcutaneously injected with pontamine blue ~ 4 days prior to collection. Red blood cells were lysed using OptiLyse and cell suspensions mounted on glass slides with DAPI-containing hard mount solution. Images were taken using rhodamine (A) and DAPI (B) filters and phase contrast (A-C). Composite images using phase contrast with rhodamine (A), DAPI (B) or rhodamine and DAPI together (C) were produced. Open arrows show neutrophil-like cells whilst closed arrows show cells with less granular nuclei such as seen in monocytes.

Figure 3.21 Pontamine blue staining at the site of injection 14 days after i.m injection site with free Ag85B-ESAT-6 (A), DDA liposomes adsorbing Ag85B-ESAT-6 (B) or DDA:TDB liposomes adsorbing Ag85B-ESAT-6 (C).

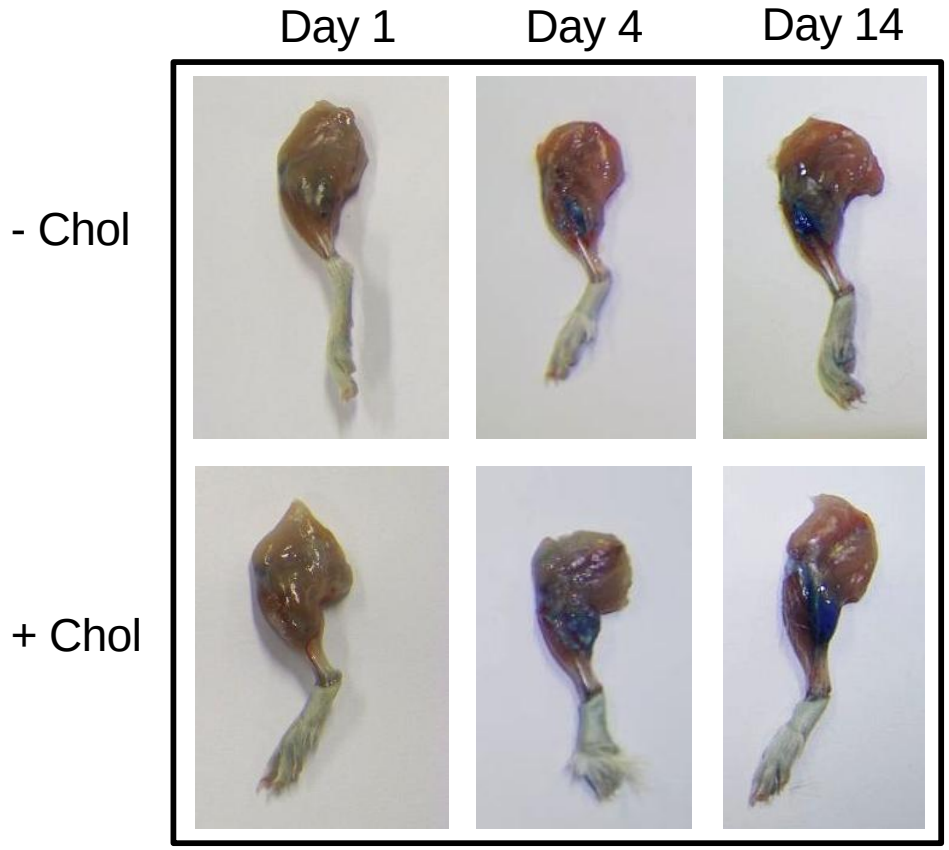

Figure 3.22 Cholesterol inclusion in DDA:TDB liposomes has no effect of the intensity of blue staining seen at the site of injection. Pictures are representative of 4 mice/group and show the quadriceps muscle from the vaccine injected leg.

3.4.8 Immunisation studies using OVA as a model antigen

DDA:TDB liposomes have been shown to elicit strong cell-mediated immunity characterised by high levels of IFN-γ, Il-2 and IgG2a production. Due to their successful track-record, DDA:TDB liposomes were chosen as the ideal formulation to investigate inter-researcher variability and the effect of various routes of administration on the observed immune responses. OVA antigen was used as a model antigen for both studies as it is well characterised and allows for a direct comparison between the two studies. Regarding the experiment to investigate inter-researcher variability based at Aston University, all three researchers had experience formulating DDA:TDB liposomes and whilst only a limited number of mice were allocated per researcher (1 or 2 per experimental group, see Chapter 2, Table 2.4), the aim was that final results could be collated to provide statistically relevant data. The second study involved the collaboration with Pål Johansen, University Hospital of Zurich, looked at the effect of the administration route in addition to addressing whether omission of the immunomodulator TDB from DDA liposomes affected immunogenicity.

The results derived from the immunisation study conducted at Aston University to investigate inter-person variability were very mixed. Whilst a successful antibody response with little inter-person variation was observed, the results relating to re-stimulated splenocyte proliferation and their cytokine production were disappointing. Figure 3.23 shows total IgG (A) and IgG1 (B) antibody production in response to immunisation with OVA alone, adsorbed to DDA:TDB liposomes or mixed with Incomplete Freund's Adjuvant (ICF). All mice injected with DDA:TDB:OVA liposomes mounted a strong IgG response which peaked 35 days after the initial immunisation. By contrast, ICF:OVA injected mice required longer for all mice to mount a similarly strong IgG response. Injection of OVA alone did also cause increases in the IgG titre however these were lower at each time-point compared to adjuvanted formulations. With regards to the IgG1 response, immunisation of OVA with DDA:TDB liposomes, but not with ICF or alone, induced above background levels of IgG1 in all 6 mice after just one immunisation (Figure 3.23, B). By contrast, OVA immunisation with ICF required one booster injection whilst OVA administered alone required two booster injections. Neither group however reached similar levels of IgG1 production as seen after immunisation with DDA:TDB liposomes. Naïve mice did not mount any IgG response. In addition to testing mouse sera for IgG1, the

IgG2a response was measured but no IgG2 production was noted (results not shown). Increasing the concentration of IgG2 antibody and decreasing the serum dilutions had no effect. These results were unexpected as DDA:TDB liposomes have previously been shown to elicit IgG2 antibody production when given both in conjunction with the TB antigen Ag85B-ESAT-6 (Davidsen et al., 2005; Kirby et al., 2008a; Kirby et al., 2008b) and with OVA (Agger et al., 2008b) (although OVA leads to IgG2a responses ~ 10-fold lower than Ag85B-ESAT-6). This disparity between the documented production of IgG2 antibodies in mice immunised with DDA:TDB liposomes compared to the nonexistent IgG2 responses noted here could have been due to a number of factors including the antigen dose, the use of OVA antigen as opposed to the strongly antigenic Ag85B-ESAT-6 antigen and the choice of mouse strain (Balb/c versus the more traditionally used C57Bl/6). These variables were addressed in a subsequent immunisation study in which C57Bl/6 mice were immunised with Ag85B-ESAT-6 antigen (Chapter 6).

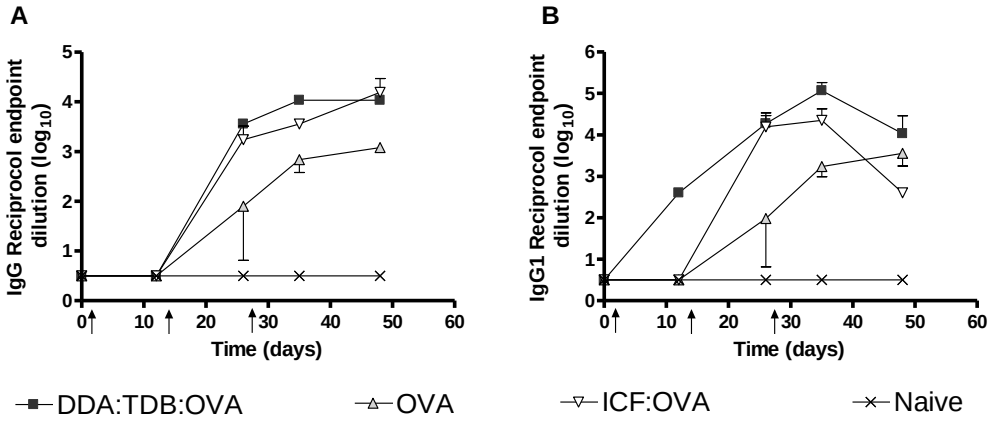

Figure 3.23 Antibody responses to immunisation with OVA alone, with DDA:TDB liposomes or with ICF. Total IgG (A) and IgG1 (B) are shown as the $\log_{10}$ of reciprocal endpoint 3-fold dilutions (starting at 1:400) required to reach an OD of 0.2 derived from the mean ± SD of 6 mice (DDA:TDB:OVA and OVA groups) or 3 mice (ICF:OVA and naïve groups). Each mouse (Balb/c) received 3 injections on days 1, 14 and 28 (shown with arrows).

DDA and DDA:TDB liposomes were also sent to Pål Johansen, University Hospital of Zurich, where the effect of the route of injection was investigated, in addition to a comparison between different vesicular systems as adjuvants for OVA immunisation. OVA (20 µg/dose) and Balb/c mice were again used thereby providing a comparative study to the OVA-immunisation study conducted at Aston University. Figure 3.24 shows the IgG1 and IgG2a responses to DDA and DDA:TDB liposomes. In accordance with the results obtained at

Aston University, DDA-based liposomes did not need a booster injection to elicit IgG1 responses, regardless of the route of injection used or whether TDB was included in the formulation (Figure 3.24 A, B). Initially there were significantly higher levels of IgG1 after administration of DDA or DDA:TDB liposomes via the i.ln route as opposed to the i.d, i.m or s.c routes. These differences diminished however upon administration of a booster injection on day 39, after which IgG1 titres were greatly improved and reached peak titres of above 200,000 regardless of the immunisation route.

Figure 3.24 The effect of route of administration on the antibody response against DDA (A, C) and DDA:TDB (B, D) liposomes adsorbing OVA. Liposomes were injected into Balb/c mice on days 0 and 39 (depicted by arrows) via the intralymphatic (i.ln), intradermal (i.d), intramuscular (i.m) or subcutaneous (s.c) routes of administration. Serum was collected at relevant time-points and analysed for the presence of IgG1 (A, B) and IgG2a (C, D) antibodies. Antibody titres are expressed as the inverse of the highest dilution reaching an absorbance equal or higher than that of a negative serum plus two standard deviations, and the titres were expressed as means ± SD (n = 4). Previously published (Mohanan et al., 2010).

IgG2a responses were noted but these were more dependent on the booster injection given on day 39. Whilst DDA liposomes did not induce any significant levels of IgG2a prior to the day 39 booster, regardless of the injection route, after this booster injection the

IgG2a titres detected after i.ln or i.d immunisation reached ~ 100,000. Only very small increases in the IgG2a titres were noted after i.m and s.c booster injections of DDA liposomes (Figure 3.24, C). Inclusion of TDB in the DDA liposome formulation gave a very different result with i.ln, i.d and i.m injection all reaching peak titres of ~ 10,000 – 100,000 by the end of the study. Only DDA:TDB injection via the s.c route did not cause significant production of IgG2a.

From these results it appears that both the i.ln and i.d routes of injection offer the fastest and strongest immune response characterised by both IgG1 and IgG2a production. Omission of TDB from DDA liposomes does not appear to have a major effect on the IgG1 or IgG2a responses noted, with the exception of IgG2a levels after i.m injection. As to why DDA:TDB liposomes caused IgG2a production in this experiment whilst no IgG2a was seen in the experiment conducted at Aston University is unclear. In both experiments the vaccines were formulated by the same person using the same raw materials, with the only differences in the immunisation protocol being the antigen dose and the immunisation timescale (3 doses with 2-week intervals verses 2 doses with a 39-day interval). Furthermore, the concentration of OVA antigen used for IgG2a detection (via indirect ELISA) was 10-fold higher for the Aston University immunisation experiment, suggesting that the problem was not related to the IgG2a detection protocol. To test this, the sera from DDA:TDB:OVA immunised mice was tested using antibody isotype testing strips (AbDSerotec, Oxford, UK) which offer a fast and reliable method to detect antibodies (IgG1, IgG2a, IgG2b, IgG3, IgM and IgA) in mouse serum. The results show that little if any IgG2a was present in the sera of DDA:TDB:OVA immunised (and naïve) mice (Figure 3.25) and therefore confirms the previous suggestion that the lack of detection of IgG2a by ELISA was actually due to no IgG2a production by the mice.

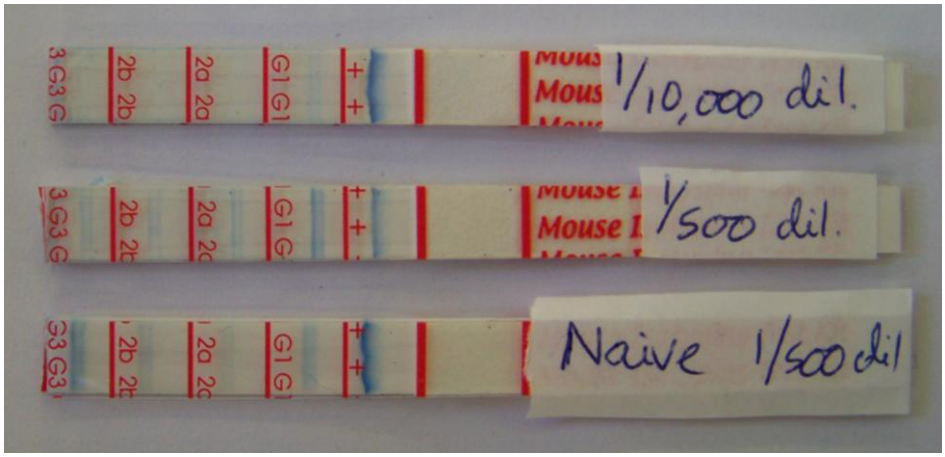

Figure 3.25 Isotyping test strips in which sera from DDA:TDB:OVA immunised mice at a 1/10,000 and 1/500 dilution was tested for the presence of IgG2a. Sera from unimmunised mice (1/500 dilution) is also shown for comparison. A blue line shows the presence of the corresponding antibody whilst a blue line next to the ' + + ' marker confirms the strip efficacy. Whilst IgG1 antibody is present in all samples shown, only low levels of IgG2a can be detected.

In addition to assessing the antibody responses, spleens from mice were restimulated *in vitro* with OVA to investigate splenocyte proliferation and cytokine responses. The results obtained from the Aston immunisation study were highly dependent on the researcher conducting the splenocyte preparation. Figure 3.26 shows the proliferative responses of splenocytes from mice injected with OVA alone, OVA combined with ICF or DDA:TDB liposomes and finally naïve PBS injected mice. All splenocyte cultures were restimulated with ConA as a positive control, however, as is seen in Figure 3.26, A, not all splenocyte cultures responded to the positive control. Looking specifically at splenocytes processed by MH (Figure 3.26, B), all immunised mice responded well to ConA and showed an OVA concentration dependent increase in proliferation. DDA:TDB:OVA immunised mice showed the highest levels of proliferation of splenocytes with thymidine uptake being between 10 - 100 fold higher that that noted of OVA injected alone or with ICF. These results show that OVA immunisation with DDA:TDB liposomes leads to higher levels of splenocyte proliferation than seen with OVA alone or with the commonly used ICF adjuvant. It is important to note however that these results are only indicative of 1 or 2 mice as the results obtained from RK and JH processed mice did not respond well to ConA (Figure 3.26, A) or to OVA (results not shown). This inter-researcher variation was however only noted with assays relating to splenocytes – no variation in IgG antibody presence in the sera was noted (Figure 3.27).

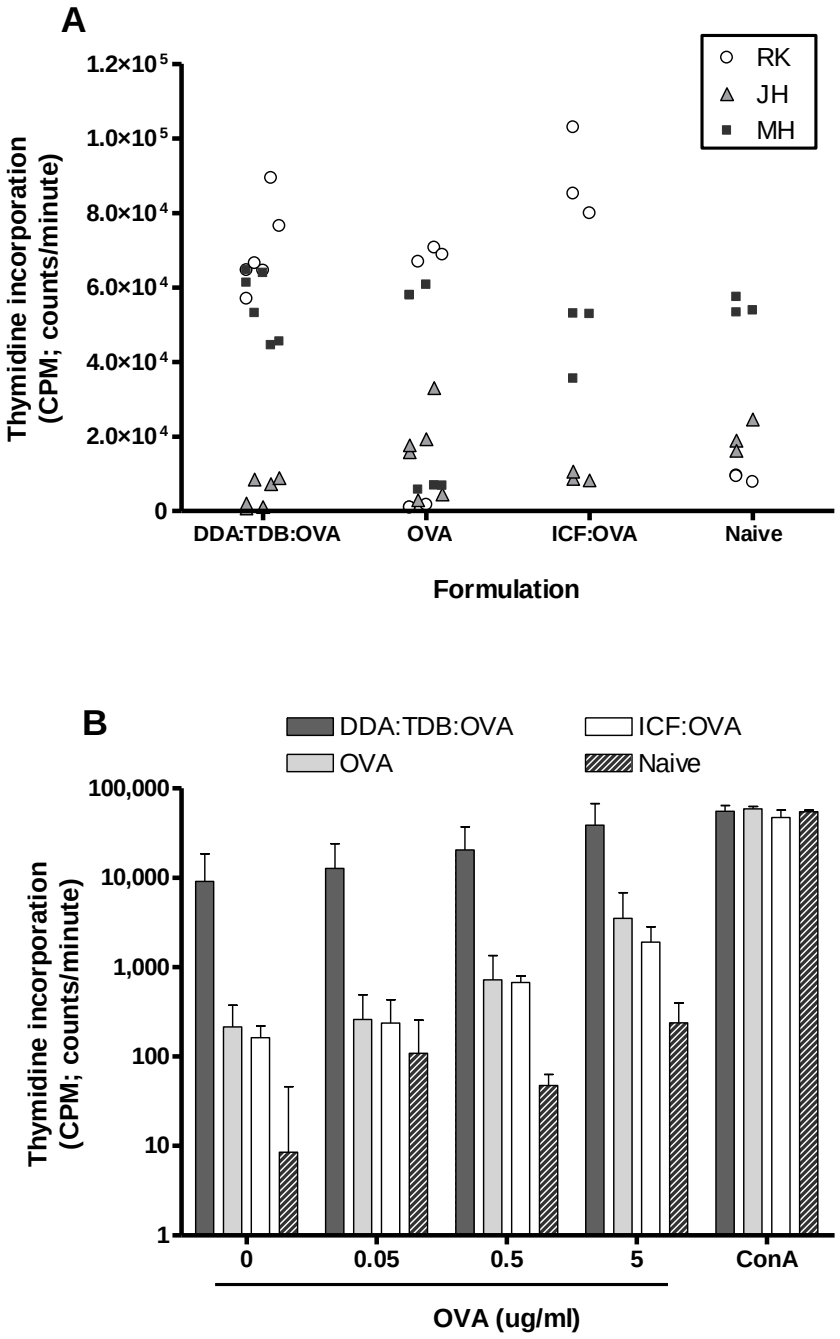

Figure 3.26 Splenocyte proliferation, as measured by tritiated thymidine uptake, in response to OVA antigen restimulation or exposure to ConA. A; Splenocytes derived from mice immunised with DDA:TDB:OVA liposomes, OVA alone, ICF:OVA or PBS were exposed to the positive control stimulant ConA (1 µg/ml) and the level of proliferation measured using tritiated thymidine uptake expressed in counts per minute (CPM). Splenocytes from 6 (DDA:TBD:OVA and OVA) or 3 mice (ICF:OVA and naïve) were investigated in triplicate – results show thymidine uptake of individual wells with the results of each researcher (RK, JH or MH) represented. B; Splenocytes derived from mice immunised with DDA:TDB:OVA liposomes, OVA alone, ICF:OVA or PBS (naïve) were exposed to the positive control stimulant ConA (1 µg/ml) or OVA at concentrations of 0.05, 0.5 or 5 µg/ml. Results are the mean ± SD of triplicate wells of mice injected and processed by MH only.

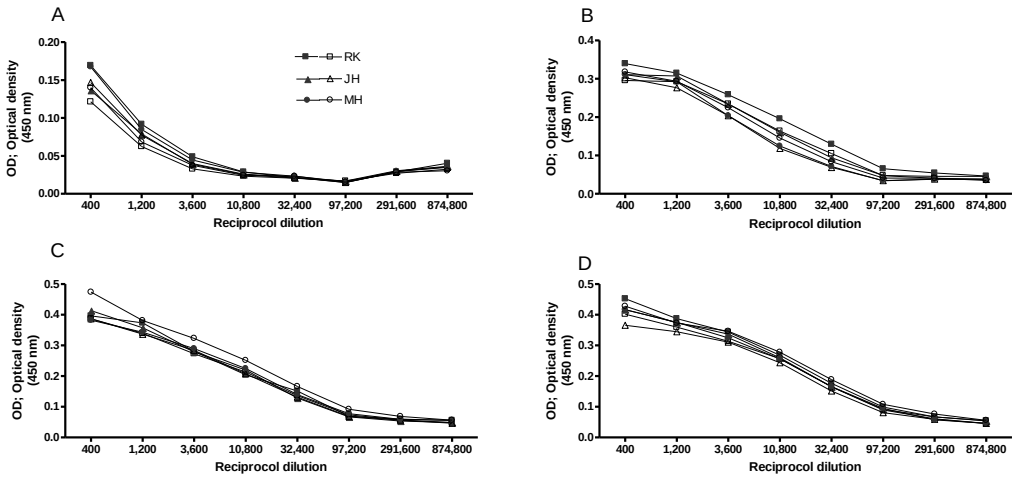

Figure 3.27 Total IgG OD readings for serially diluted serum from DDA:TDB:OVA injected mice (n=6). Blood was collected on days 12, 24, 35 and 48 (A, B, C and D respectively) post injection of DDA:TDB liposomes adsorbing OVA. Each investigator (■/□ RK, Randip Kaur; ▲/Δ JH, Jubair Hussain; ●/○ MH, Malou Henriksen) made and injected the liposomes and collected serum from their respective mice (1-6).

In addition to looking at splenocyte proliferation, the supernatants of restimulated splenocytes were collected and assayed for the presence of various cytokines (again, only MH processed splenocytes were investigated). The data obtained from [MH processed] splenocytes was rather unexpected as whilst ConA simulated splenocytes produced relatively high levels of all cytokines measured, cytokine production in response to OVA was low (Figure 3.28). All DDA:TDB:OVA and ICF:OVA immunised mice were capable of producing cytokines in response to ConA to levels of ≥ 300 pg/ml (Figure 3.28, A). OVA immunised mice were not highly responsive to ConA as less than ~ 300 pg/ml of all cytokines was detected. With regards to the responses of DDA:TDB:OVA immunised mice to OVA specifically, the results were unexpected; levels of IL-2 and IFN-γ were low/non-existent with many non-responsive cultures even though both these cytokines, representative of Th1 immune responses, have previously been shown to be produced in high amounts after DDA:TDB immunisation (Kirby et al., 2008a; Vangala et al., 2007). With regards to IFN-γ in particular, the results are highly surprising as DDA:TDB liposomes have an established history of being capable of inducing high levels of IFN-γ in splenocyte and blood cultures (Agger et al., 2008b; Christensen et al., 2010a; Davidsen et al., 2005; Holten-Andersen et al., 2004; Kirby et al., 2008a; Linderstrøm et al., 2009; Vangala et al., 2007). However, it is important to note that none of these immunisation studies used OVA as an antigen, instead preferring disease antigens such as Ag85B-ESAT-6 or hepatitis A

antigen. Importantly, IFN-γ production in TB vaccine studies can be directly correlated with vaccine efficacy (Agger and Andersen, 2001) and therefore it is an important cytokine to measure in such studies. Levels of IL-10 were also low however IL-5 and IL-6 production appeared to show an OVA restimulation concentration dependent effect with splenocytes derived from DDA:TDB:OVA immunised mice producing the highest levels of IL-5 (~ 150 pg/ml for both). IL-5 is a typical Th2 cytokine and is expressed to variable levels in DDA:TDB vaccine studies. However, in the majority of cases the levels are low and occasionally not significantly higher that background naïve mice (Kirby et al., 2008a; Vangala et al., 2007). IL-6 is also thought of as a Th2 cytokine and has a role in directing $CD4^+$ T cell differentiation into Th2 cells via upregulation of IL-4 production and suppression of IFN-γ signalling and production (Diehl and Rincón, 2002; Dienz and Rincon, 2009; Rincón et al., 1997). In an interesting twist, although IL-6 shifts the Th1/Th2 balance towards the Th2 immune response, IL-6 has also been shown to promote differentiation of Th17 cells with production of IL-17 (Dienz and Rincon, 2009). This is especially interesting with regards to DDA:TDB vaccination as DDA:TDB liposomes have recently been shown to induce high amounts of IL-17 after vaccination with Ag85B-ESAT-6 (Henriksen-Lacey et al., 2010c; Kamath et al., 2009). Therefore IL-6 may act in counterproductive ways in this current vaccination model.

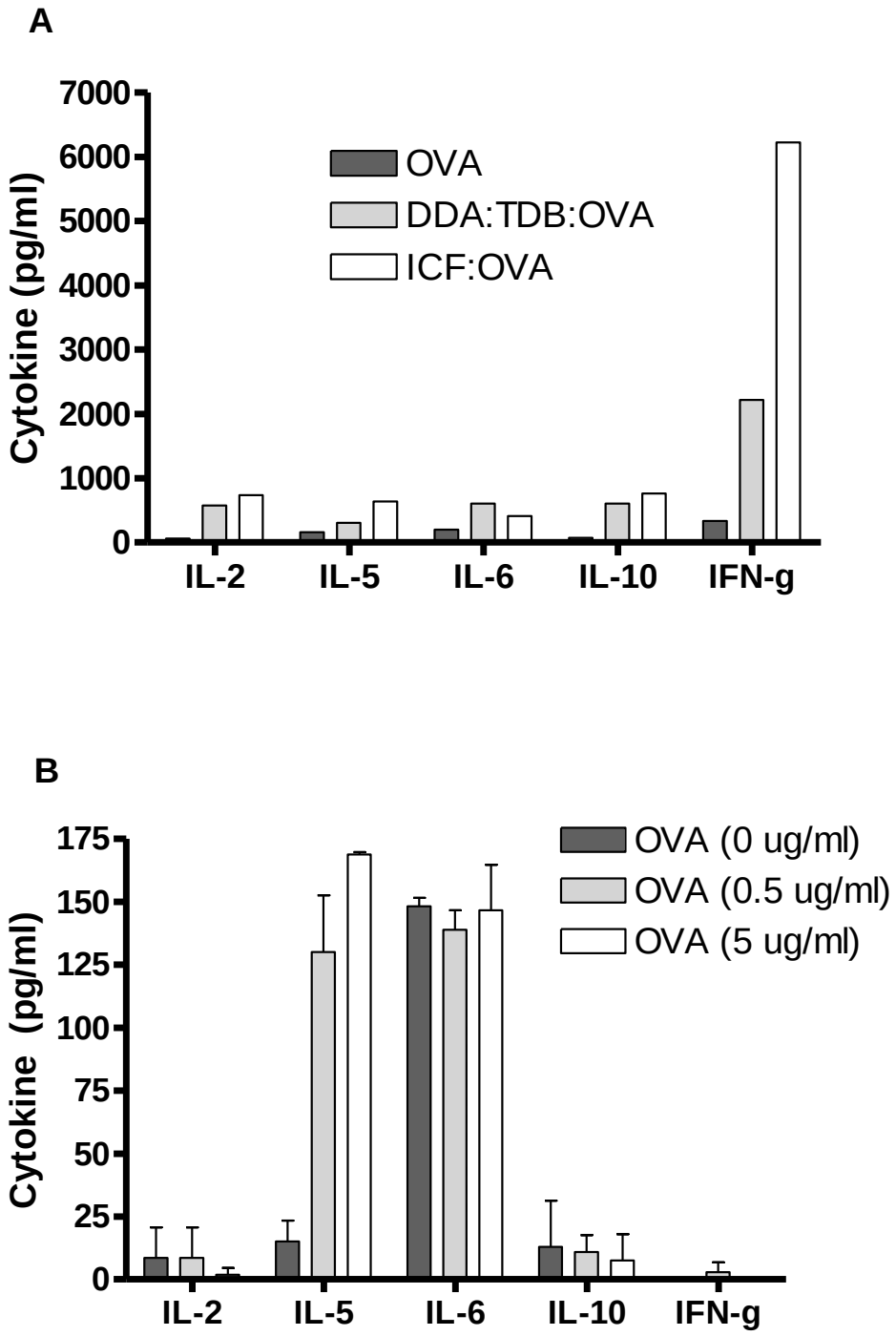

Figure 3.28 Cytokine production from ConA (A) or OVA (B) restimulated splenocytes derived from [MH] immunised mice. A; Splenocytes derived from mice immunised with OVA alone, DDA:TDB:OVA or ICF:OVA were restimulated in vitro with ConA (1 µg/ml) as a positive control for cytokine production. Results are representative of the mean of duplicate wells of splenocytes derived from one mouse. B; Cytokine production from splenocytes derived from MH processed DDA:TDB:OVA immunised mice and restimulated in vitro with 0, 0.5 or 5 µg/ml OVA. Results denote mean ± SD of duplicate wells of 2 mice.

Whilst IL-4 was not investigated in the study conducted at Aston University, IL-4 (and IFN-γ) production from restimulated splenocytes derived from DDA and DDA:TDB injected mice were investigated with our collaborative partners at University Hospital of Zurich. IL-4 production was low for all injection routes and did not vary significantly after the incorporation of TDB (Figure 3.29). Similar findings were found for IFN-γ secretion with the exception of the i.ln injection route with produced high levels (~ 400 pg/ml) of IFN-γ which increased ~ 1.5 fold upon incorporation of TDB into the liposomal vaccines (Figure 3.30). The successful production of IFN-γ after the i.ln injection route correlates with the strong lasting IgG1 and IgG2a response seen in the same study. However it should be noted that i.ln injection was originally included in this study as a positive control as direct application of antigen (OVA) and adjuvant to the lymphoid organ is obviously the easiest and most efficient way to expose APCs to antigen, as well as priming lymphocytes (Johansen et al., 2010). Therefore, whilst it is not really possible to compare responses between i.ln injection and other routes, it is possible to compare how the addition of TDB to the liposomal formulation affects the immune response.

Figure 3.29 IL-4 production from restimulated splenocytes obtained from mice injected with DDA:OVA liposomes (clear bars) or DDA:TDB:OVA liposomes (black bars). The effect of different injection routes was studied (i.ln, intralymphatic; i.d, intradermal; i.m, intramuscular; s.c, subcutaneous) after injection on day 1 and 39; splenocytes were harvested on day 100 and exposed to OVA (10 μg/ml) for 72 hrs prior to harvesting supernatants and detection of IL-4 by ELISA. Previously published (Mohanan et al., 2010).

Figure 3.30 The route of administration has an effect on IFN-γ production from restimulated splenocytes derived from mice immunised with DDA (A) or DDA:TDB (B) liposomes adsorbing OVA antigen. Mice were given one booster injection on day 39 and spleens removed on day 100 and restimulated with OVA (10 µg/ml). Culture supernatants were harvested after 72 hrs and analysed by ELISA for IFN-γ. Routes of administration included intralymphatic (i.ln), intradermal (i.d), intramuscular (i.m) and subcutaneous (s.c). Previously published (Mohanan et al., 2010).

3.5 Discussion and Conclusion

These initial findings confirm many of the previously documented physicochemical characteristics of DDA-based liposomes. DDA liposomes, with or without the addition of the immunomodulator TDB (11 mol %) are between 400 - 500 nm in size and express a highly cationic surface charge of ~ 40 mV. Whilst addition of TDB can enhance DDA liposomes stability, cholesterol can also be incorporated into the lipophilic bilayer although this does not counteract the unstable nature of DDA:TDB liposomes hydrated with PBS buffer. The nature of PBS-induced instabilities is due to the high saline content – reducing the molarity of PBS buffer is able to reduce vesicle aggregation.

Proteins such as OVA, BSA and Ag85B-ESAT-6, all of which express a pI of below 7 units can be effectively adsorbed to the surface of DDA-based liposomes. Whilst low doses (~ 10 μg/ml) of protein do not cause any significant physicochemical changes to DDA-based liposomes, increasing the protein dose has a significant effect resulting in aggregation of vesicles and a decrease in the zeta potential. The ability of DDA-based liposomes to adsorb Ag85B-ESAT-6 allows the resulting liposomal formulation to be used *in vivo* as an effective antigen delivery system. Upon administration via either the i.m or s.c routes of injection a strong retention of antigen at the injection site is noted and unlike injection of Ag85B-ESAT-6 alone, antigen can still be strongly detected 14 days p.i.

Liposomes composed of DDA with or without TDB can be used as adjuvants for immunisation of the model protein OVA. Whilst there is no doubt that IgG1 antibodies can be successfully induced after a single injection of DDA or DDA:TDB liposomes, the induction of IgG2 antibodies was more variable; in one study there was no production of IgG2 whilst in another its production required a booster injection. Furthermore, the characteristically Th1 cytokine IFN-γ, often linked to IgG2 production, was notably absent in both immunisation studies. Liposome administration via i.d, s.c or i.m routes of injection had no effect in the induction of IFN-γ.

Whilst the results obtained from the physicochemical characterisation of DDA-based liposomes provide some interesting findings, especially with respect to PBS induced characteristics, the finding that DDA:TDB liposomes are unable to induce the Th1 arm of the immune response when used as an adjuvant for OVA are surprising. There are many documented studies of DDA-based liposomes inducing strong levels of IFN-γ, IL-2 and IgG2, all of which are described as being characteristic of the Th1 response. However, the combination of OVA antigen and the Balb/c mouse strain is rarely used in the DDA-based liposome immunisation model. It is possible that this mouse strain is unable to mount a strong Th1 immune response against OVA when adjuvanted with DDA-based liposomes. For this reason, and presented in Chapter 6, the more commonly used C57Bl/6 mouse strain in combination with Ag85B-ESAT-6 antigen was used to investigated the immunising abilities of DDA:TDB liposomes.

Chapter 4: The Importance of Protein Antigen Adsorption to Charged Liposomes with regards to the Efficiency of Liposomes as Vaccine Adjuvants

Papers relating to this chapter

Henriksen-Lacey M., Christensen D, Bramwell V.W, Lindenstrøm T, Agger E.M, Andersen P and Perrie Y. Liposomal cationic charge and antigen adsorption are important properties for the efficient deposition of antigen at the injection site and ability of the vaccine to induce a CMI response. 2010, Journal of Controlled Release 145, 102-108.

4.1 Aims

The aim of the work reported in this chapter was to investigate the importance of liposome-antigen interactions, defined by electrostatic association, for the successful production of an adjuvant and antigen depot-effect at the site of injection. Cationic and neutral liposomes with various antigens were studied and their physicochemical characteristics noted. Biodistribution and immunogenicity studies of certain formulations were undertaken and collaborations involving dendritic cell activation studies were pursued.

4.2 Introduction

Cell bilayers express an overall anionic surface charge due to the presence of anionic lipids phosphatidylserine (PS) and phosphatidylinositol (PI), in combination with the zwitterionic lipids phosphatidylcholine (PC) and sphingomyelin (SM) (Ohvo-Rekilä et al., 2002). Upon *in vivo* administration of cationic liposomes, electrostatic attractions between the cell surface and liposomes can occur, unrelated to the presence of receptor-ligand interactions. Whilst this is one of the properties which makes cationic liposomes efficient transfection agents (Gao and Huang, 1991; Kawakami et al., 2004; Li et al., 2010; McNeil and Perrie, 2006; Ramezani et al., 2009; Simberg et al., 2004), there are also reports that anionic cell surface glycosaminoglycans can actually inhibit cationic liposome mediated gene uptake (Ruponen et al., 2004). Prior to the use of liposomes as transfection agents, liposomes were used primarily as drug delivery systems, however, problems with drug leakage, liposome instability and rapid clearance via the mononuclear phagocyte system (MPS) were significant problems (Gregoriadis, 1985, 1995b). Cholesterol incorporation, as well as the creation of 'stealth' liposomes by adding poly (ethylene glycol) (PEG) to the liposome surface, has been used to overcome these issues thereby minimising the binding of plasma proteins to the liposome surface (Gregoriadis, 1985; Gregoriadis and Davis, 1979).

Although plasma proteins make up less than 4.4 % of the total blood volume (the remaining being water, ~ 49.5 %, erythrocytes, ~ 45 % and (in)organic substances, ~ 1.1 %) (Sigma-Aldrich Co., 2010), they play a major role in charge-mediated liposome-aggregation. As noted in Chapter 3, the exposure of cationic liposomes to conditions which simulate the *in vivo* environment (i.e. ~ 50 % serum at 37 °C) results in rapid

aggregation of the cationic vesicles due to electrostatic interactions between serum proteins and positively charged liposomes (Chapter 3, Figure 3.9). Therefore it was questioned whether electrostatic interactions between cationic liposomes and cells or serum proteins mediate vesicle aggregation and may be the reason why such high levels of liposome deposition at the injection site, regardless of the injection route, are noted (Chapter 3, Figures 3.16 and 3.18). To investigate this, a comparison was made between cationic and neutral/anionic liposomes, the later being less likely to associate electrostatically with cells or with plasma proteins at the site of injection. The lipid distearolyphosphatidylcholine (DSPC) was used in place of DDA to form liposomes with TDB. DSPC has a zwitterionic phosphate head group and consequently forms neutral/slightly anionic liposomes. DDA:TDB and DSPC:TDB liposomes were investigated for their ability to associate with, and promote a depot-effect of, the TB antigen Ag85B-ESAT-6. Furthermore the immune response towards Ag85B-ESAT-6 delivered with DDA:TDB or DSPC:TDB liposomes was analysed.

Ag85B-ESAT-6 is itself immunogenic and has shown promising results in numerous *in vivo* studies when combined with a delivery system (Agger et al., 2008a; Agger et al., 2006; Andersen et al., 2009; Bhowruth et al., 2009; Davidsen et al., 2005; Kamath et al., 2009; Kamath et al., 2008a; Kamath et al., 2008b; Korsholm et al., 2010; Linderstrøm et al., 2009; Rosenkrands et al., 2005). Unlike the BCG vaccine which is considered the current TB vaccine gold standard, Ag85B-ESAT-6 comprises the 'ESAT-6' epitope which has been deleted in BCG due to years of sub-passage and attenuation (Agger and Andersen, 2002). Fusion of Ag85B (strong antigen) with ESAT-6 (weak antigen) to form Ag85B-ESAT-6 leads to a synergistic increase in immune responses and importantly, when combined with cationic liposomes, levels of protection equal to vaccination with BCG are conferred (Brandt et al., 2000; Olsen et al., 2001). Synergistic effects when BCG and Ag85B-ESAT-6 are combined have also been observed (Dietrich et al., 2007).

The importance of antigen association to liposomes was studied with emphasis on the liposome-mediated antigen depot-effect (described in Chapter 3). To achieve this, protein antigens of varying isoelectric points (pI) were selected based on the overall charge expressed when at neutral pH values. OVA and Ag85B-ESAT-6 both have pI values of ~ 4.5 units and therefore at neutral pH they express an overall anionic surface charge. Both antigens are able to strongly associate with cationic liposomes due to the opposing electric

charge. In contrast, it was hypothesised that an antigen with a pI of >7.4, and therefore of cationic charge above neutral pH values, would not be able to associate electrostatically with cationic liposomes.

To this aim, two proteins were tested:

- lysozyme was investigated as a model antigen as it has a high pI (11 units). Lysozyme is a 16 kDa protein and whilst not commonly used in vaccine studies, lysozyme is a useful protein to study the effect of non-electrostatically adsorbed protein to DDA based cationic liposomes.
- a 2-part subunit Chlamydia antigen termed CTH1 was also investigated as it is immunogenic and expresses an overall pI of 9 units. Itwas therefore postulated to not adsorb to cationic liposomes.

A final component in the investigation between the effects of neutral DSPC:TDB and cationic DDA-based liposomes involved a collaboration with Trinity College Dublin (The University of Dublin, Ireland) to look at the ability of these formulations to cause IL-1α and IL-1β production in dendritic cells (DCs).

4.3 Results and Discussion

4.3.1 Physicochemical characteristics including characterisation of protein association

Liposomes composed of DDA and TDB at a 5:1 weight ratio expressed similar physicochemical characteristics as described in Chapter 3. DSPC:TDB liposomes were made at 5:1 weight ratios and 8:1 molar ratios (therefore mimicking the DDA:TDB formulation) without any change in the physicochemical characteristics noted: DSPC:TDB liposomes had an average vesicle size 1488 nm and polydispersity of 0.381 (Table 4.1). DSPC:TDB liposomes were approximately 3-fold larger than their counterpart DDA:TDB liposomes (Table 4.1). This size variation was confirmed by TEM imaging and by inclusion of trace quantities of rhodamine labelled DPPC and fluorescence imaging (Figure 4.1). DSPC lipid has a zwitterionic head group and therefore liposomes produced of DSPC with TDB express a neutral/slightly anionic surface charge. Therefore, substitution of DDA with DSPC results in a decrease in surface charge from approximately +50 mV to -5 mV (Table 4.1).

Table 4.1 Physicochemical characteristics of DDA:TDB and DSPC:TDB liposomes

	DDA:TDB Liposomes	DSPC:TDB Liposomes
Vesicle diameter (nm)	450 ± 63	1488 ± 239
Polydispersity	0.282 ± 0.037	0.381 ± 0.024
Zeta potential (mV)	46 ± 7	- 4.1 ± 9

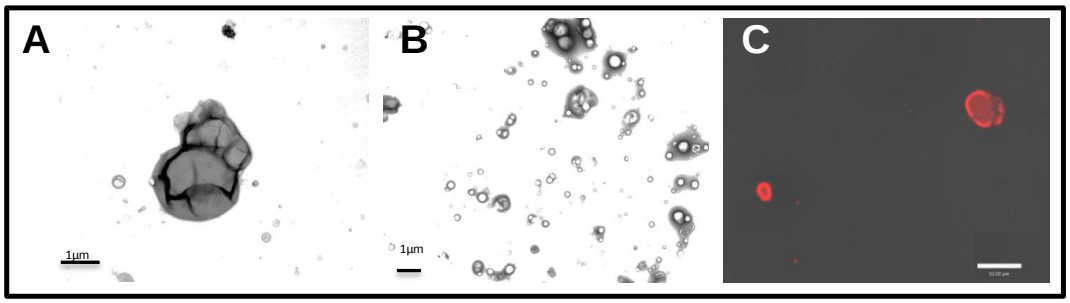

Figure 4.1 TEM (A, B) and fluorescence (C) images of DSPC:TDB liposomes showing their supra-micron size and multilamellar nature.

The effect of protein on the physicochemical characteristics of DDA:TDB and DSPC:TDB liposomes was investigated initially using the model protein OVA as high concentrations of protein could be added to the liposomes. OVA was dissolved in a Tris buffer (10 mM, pH 7.4) and added to DSPC:TDB and DDA:TDB liposomes to a final OVA concentration of 1, 5 or 10 mg/ml. Figure 4.2 shows the effect of OVA at these concentrations on the vesicle size and surface charge of the liposomes. Unlike the immediate and significant aggregation of DDA based vesicles upon protein addition, DSPC:TDB liposomes do not aggregate or change significantly in their physicochemical characteristics. OVA expresses an anionic charge under the pH neutral conditions used and therefore addition of OVA to the neutral DSPC:TDB liposome construct does not favour strong levels of electrostatic interaction and adsorption between the protein and liposomes (Figure 4.3). However, addition of 5 or 10 mg/ml to DSPC:TDB liposomes did lead to some association (~ 13 % of that added, therefore equivalent to between 0.65 – 1.3 mg/ml), suggesting that the DSPC:TDB liposome surface may become saturated at higher protein concentrations.

By contrast to the low OVA association to DSPC:TDB liposomes upon addition of 1 mg/ml, strong adsorption of OVA to DDA:TDB liposomes was noted and is in correlation with

previous results investigating proteins with a pI of between 4 - 5 units (Chapter 3, Figures 3.8 and 3.10).

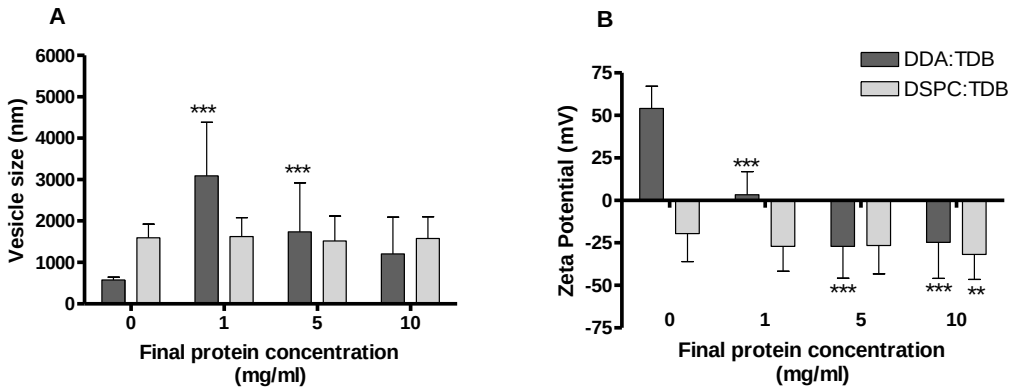

Figure 4.2 The effect of addition of OVA to DDA:TDB and DSPC:TDB liposomes made with Tris buffer. OVA was added to liposomes to a final concentration of 1, 5 or 10 mg/ml liposomes. Vesicle size (A) and zeta potential (B) were measured using a Brookhaven ZetaPlus machine. Results denote mean ± SD of 4 separate studies. Where shown, significance between data is against results of 0 mg/ml protein (**p<0.01, ***p<0.001).

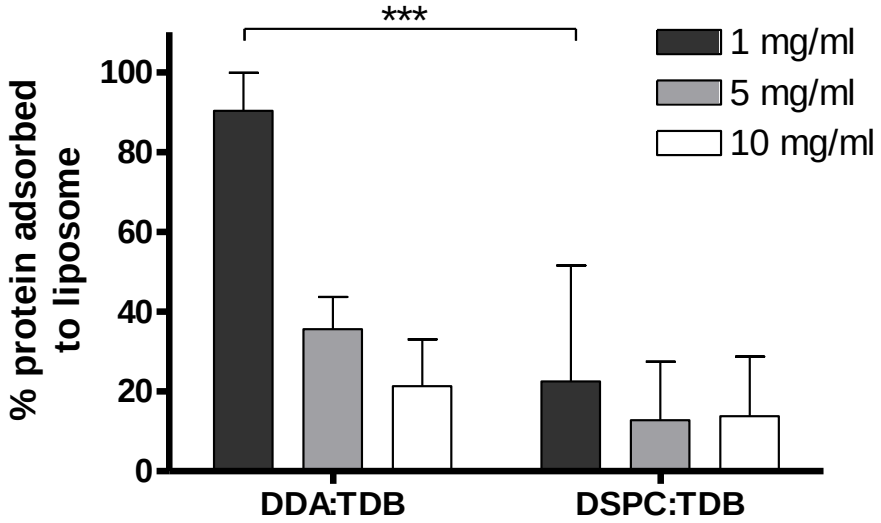

Figure 4.3 Quantification of OVA adsorption to DDA:TDB and DSPC:TDB liposomes when added to a final concentration of 1, 5 or 10 mg/ml. Protein adsorption was determined using the BCA assay and is expressed as a % of that added. Results denote mean ± SD of 4 separate studies. *n.s*, not significant; ***p<0.001.

Whilst OVA is a model protein to characterise the effects of a wide range of protein concentrations on charged liposomes, we also wished to examine model antigenic proteins as well as those with high pI values. To this aim three different proteins were

added to DSPC:TDB and/or DDA:TDB liposomes to a final concentration of 10 µg (Ag85B-ESAT-6) or 25 µg (lysozyme or CTH1) per ml liposomes. The higher doses of lysozyme and CTH1 were chosen as immunisation with CTH1 had previously been documented at 5 µg and not 2 µg/dose (Andersen et al., 2009), and as lysozyme was expected to exhibit more similarity to CTH1 than to Ag85B-ESAT-6, it too was characterised at this higher dosage.

Figure 4.4 shows the adsorptive abilities and effect of Ag85B-ESAT-6, lysozyme and CTH1 on DDA:TDB and DSPC:TDB liposomes. Whilst the addition of Ag85B-ESAT-6 (10 µg/ml) or lysozyme (25 µg/ml) to DDA:TDB or DSPC:TDB liposomes had no effect on the vesicle size or polydispersity of the formulations, addition of CTH1 (25 µg/ml) altered the vesicle size of these formulations significantly (DDA:TDB, $p<0.001$; DSPC:TDB, $p<0.01$; Figure 4.4 A, C). With regards to the protein induced changes in zeta potential, CTH1 is the only protein that significantly ($p<0.001$) altered the zeta potential of DDA:TDB liposomes (Figure 4.4, B). This effect was not seen with DSPC:TDB liposomes (Figure 4.4, D).

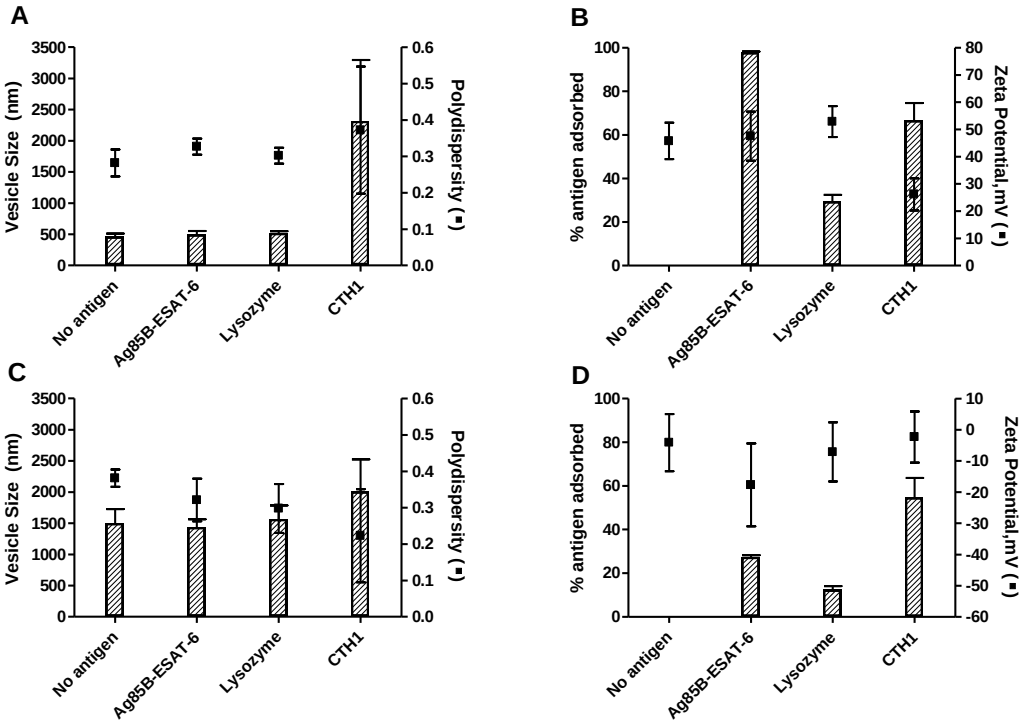

Figure 4.4: Variation in physicochemical properties of DDA:TDB (A,B) and DSPC:TDB (C,D) liposomes upon addition of the antigens Ag85B-ESAT-6 (10 µg/ml), lysozyme or CTH1 (both 25 µg/ml). The antigens were added to the liposomes post-hydration of the lipid film. Characteristics such as vesicle size (A,C; bars), polydispersity (A,C; points), zeta potential (B,D; points) and antigen adsorption to the liposomes (B,D; bars) were determined. Results represent mean ± SD of at least 3 samples.

With regard to protein adsorption, and as observed previously in Chapter 3, Ag85B-ESAT-6 bound strongly to cationic DDA:TDB liposomes (98 ± 1 %) and, as postulated due to the similarly charged nature of both components, Ag85B-ESAT-6 only weakly associated to DSPC:TDB liposomes (27 ± 1 %). As expected, the high pI value of lysozyme (11.6 units) did not lead to strong adsorption to cationic DDA:TDB liposomes due to electrostatic repulsion; only 29 % was found associated which was confirmed by SDS-PAGE (Figures 4.4 and 4.5). Low association of lysozyme to DSPC:TDB liposomes (12 %; Figure 4.4, D) was also noted and qualitatively confirmed by SDS-PAGE (Figure 4.5). However unexpectedly, CTH1 was shown to bind to both DDA:TDB and DSPC:TDB liposomes to relatively strong levels of 66 ± 8 % and 54 ± 9 % respectively (Fig 4.4 B,D and Fig 4.5). This relatively strong adsorption, combined with the effect CTH1 had on liposome size, lead to further investigations.

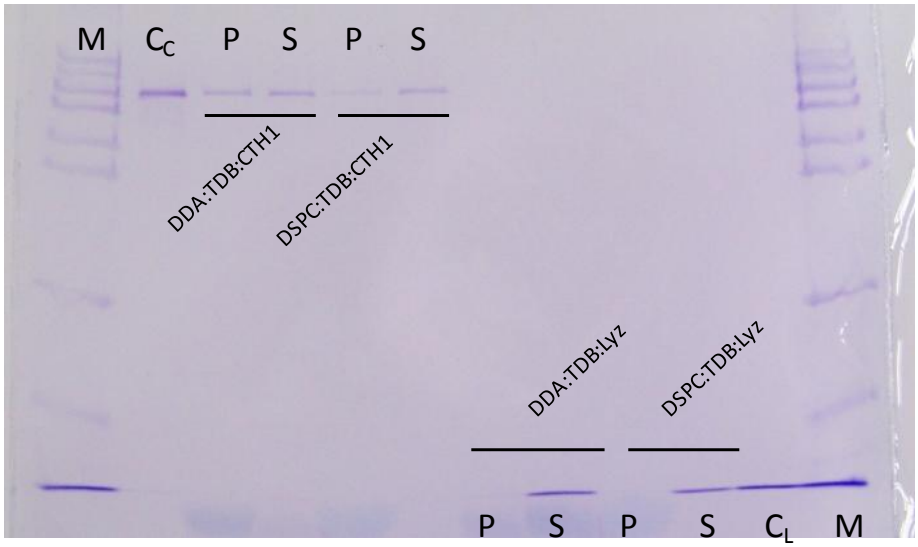

Figure 4.5 SDS-PAGE gel confirming the low/medium-binding efficiencies of lysosome and CTH1 respectively to DDA:TDB and DSPC:TDB liposomes. Proteins were added to liposomes for 1 hr to allow adsorption (if any) to occur, after which formulations were centrifuged (13 krpm, 30 mins) to separate unbound (supernatant, S) and bound (pellet, P) lysozyme and CTH1 from the liposome formulations. CTH1 and lysozyme antigen control lanes (C_c and C_L respectively) and molecular markers (M) are also shown.

CTH1 is an unstable protein and is therefore supplied in a phosphate-citrate buffer (70 mM) of low pH (pH 4) with glycerol (10 %) and cysteine (10 mM) added as stabilising agents (Dennis Christensen, personal communication). Initially the effect of the glycerol and cysteine components on the various liposome formulations were investigated.

However, their addition to DDA:TDB liposomes did not affect the physicochemical characteristics of DDA:TDB liposomes (Figure 4.6, A).

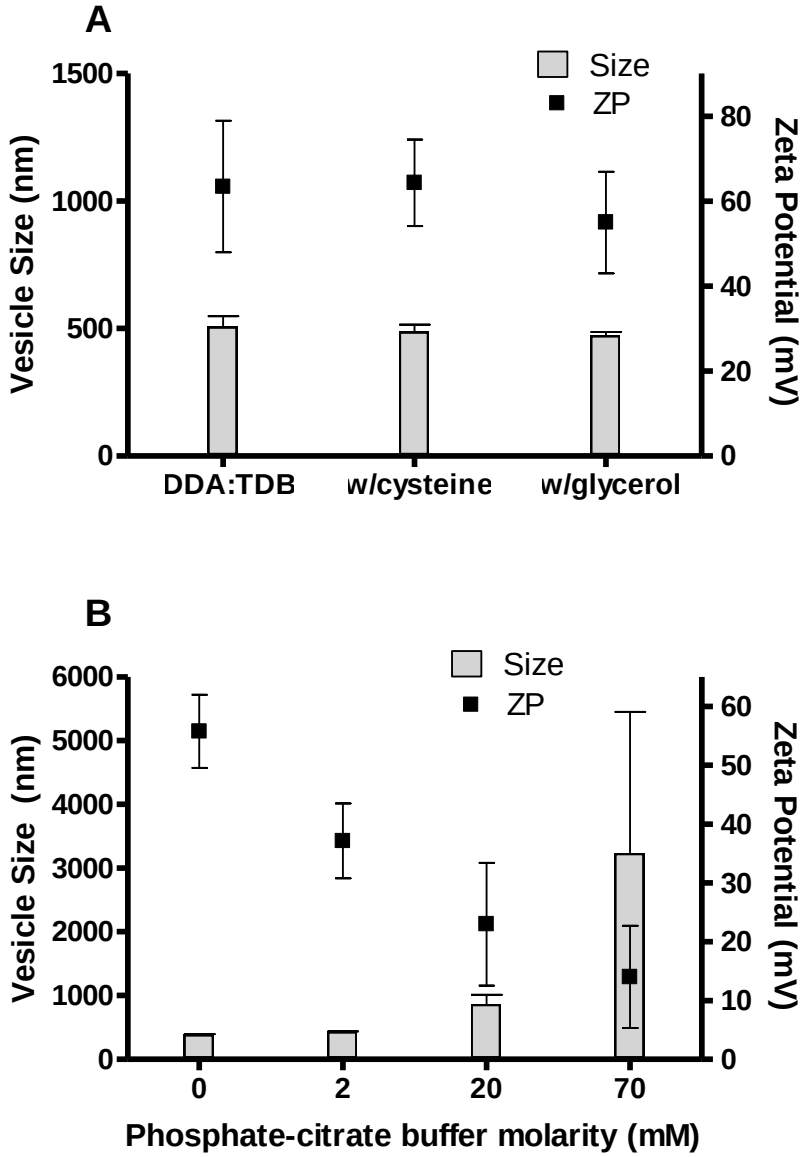

Figure 4.6 Determination of the aggregation mediating component of CTH1 on DDA:TDB liposomes. The effect of stabilising components cysteine and glycerol (A) and the molarity of the phosphate citrate buffer (B) were all investigated by measuring the vesicle size (left axis, bars) and zeta potential (right axis, points) of DDA:TDB liposomes. Results are the mean ± SD of 3 experiments.

As buffer choice and molarity had previously been shown to have an effect on liposome characteristics (Chapter 3, section 3.3.1), the effect of the phosphate-citrate buffer on the DDA:TDB liposomes was consequently studied (Fig 4.6B). Various molarities of phosphate-

citrate buffer (pH 4) were added to DDA:TDB liposomes post formation (liposomes were still hydrated in Tris buffer). The liposomes were mixed with the buffer and left for 10 min followed by size and zeta potential analysis. A trend of increasing vesicle size and decreasing in zeta potential was observed upon increasing the molarity of the phosphate-citrate buffer (Figure 4.6, B). The addition of 70 mM phosphate-citrate buffer (the same molarity as CTH1 is dissolved in) resulted in immediate aggregation of the DDA:TDB liposomes, the sizes of which were out of the range of the Brookhaven *ZetaPlus* instrument (although a value is recorded, vesicle size is measured by Brownian motion which is therefore affected if the size of particles is so large that sedimentation may occur). Therefore it was determined that the CTH1 mediated changes in vesicle size and zeta potential observed upon addition of 25 µg/ml CTH1 to DDA:TDB and DSPC:TDB liposomes was due to the phosphate citrate buffer and not the CTH1 protein itself.

4.3.2 Liposome characteristics in simulated *in vivo* conditions

In Chapter 3 the stability of DDA:TDB liposomes over a 56-day period was described (Chapter 3, Figure 3.6). These liposomes showed good stability with regards to consistent vesicle size and zeta potential measurements. Furthermore their stability over a shorter time period, but in conditions designed to simulate the *in vivo* environment, was assessed and whilst liposome aggregation was noted (Chapter 3, Figure 3.9), DDA:TDB liposomes retained Ag85B-ESAT-6 antigen well (Chapter 3, Figure 3.10). To investigate if DSPC liposomes were subject to similar aggregation, the effect of FCS on DDA:TDB and DSPC:TDB liposomes in combination with and without the three different antigens was also investigated (Figure 4.7).

Similar to the results described in Chapter 3, the exposure of DDA:TDB liposomes to FCS results in a significant and immediate aggregation of vesicles and drop in the zeta potential of ~ 35 mV regardless of the protein antigen added to the liposomes (Figure 4.7, A, B). Incubation of DSPC:TDB liposomes under the same conditions had no significant effect on either the vesicle size or zeta potential of the liposomes. These FCS induced effects were further investigated over a longer period of time using Ag85B-ESAT-6 antigen; only cationic DDA:TDB liposomes were affected by the FCS whilst DSPC:TDB liposomes remained ~ 1500 nm in size and ~ -30 mV in surface charge throughout the study (Figure 4.8, A, B). Furthermore, no changes in Ag85B-ESAT-6 adsorption were noted over time with 26 ± 9 % of the original amount of Ag85B-ESAT-6 added to DSPC:TDB liposomes

recovered after 96 hrs (Figure 4.8, C). The FCS induced changes in physicochemical properties of cationic DDA:TDB liposomes have been noted previously (McNeil and Perrie, 2006) and are due to interactions between charged plasma proteins and the positively charged surface of the liposomes. As DSPC:TDB liposomes are weakly anionic/neutral, they consequently do not interact with plasma proteins in the same way and no significant plasma protein binding to the DSPC:TDB liposomes which could cause vesicle aggregation is observed, neither is any Ag85B-ESAT-6 displaced.

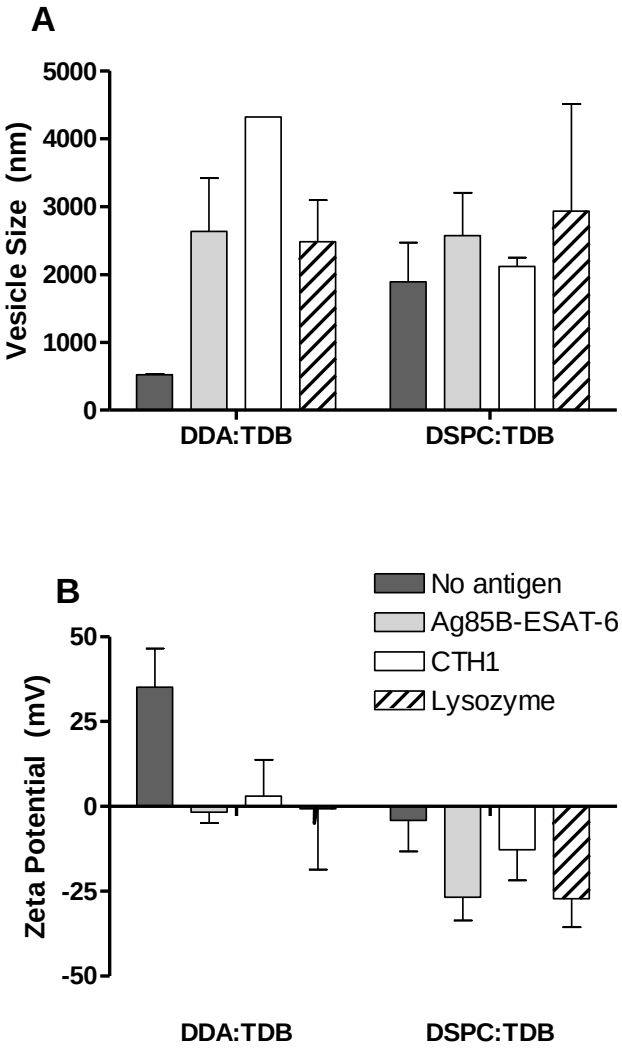

Figure 4.7 The effect of FCS on the vesicle size (A) and zeta potential (B) of DDA:TDB and DSPC:TDB liposomes without or associated with various proteins antigens. Ag85B-ESAT-6, CTH1 and lysozyme proteins were added to liposomes at dose concentrations and left to adsorb for 1 hr prior to dilution with 50 % FCS/Tris buffer, thereby simulating the *in vivo* milieu. The formulations were incubated at 37 °C for 1 hr, followed by centrifugation to obtain a pellet. The pellet was washed twice prior to measurement of the vesicle size and zeta potential. Results denote mean ± SD of triplicate experiments.

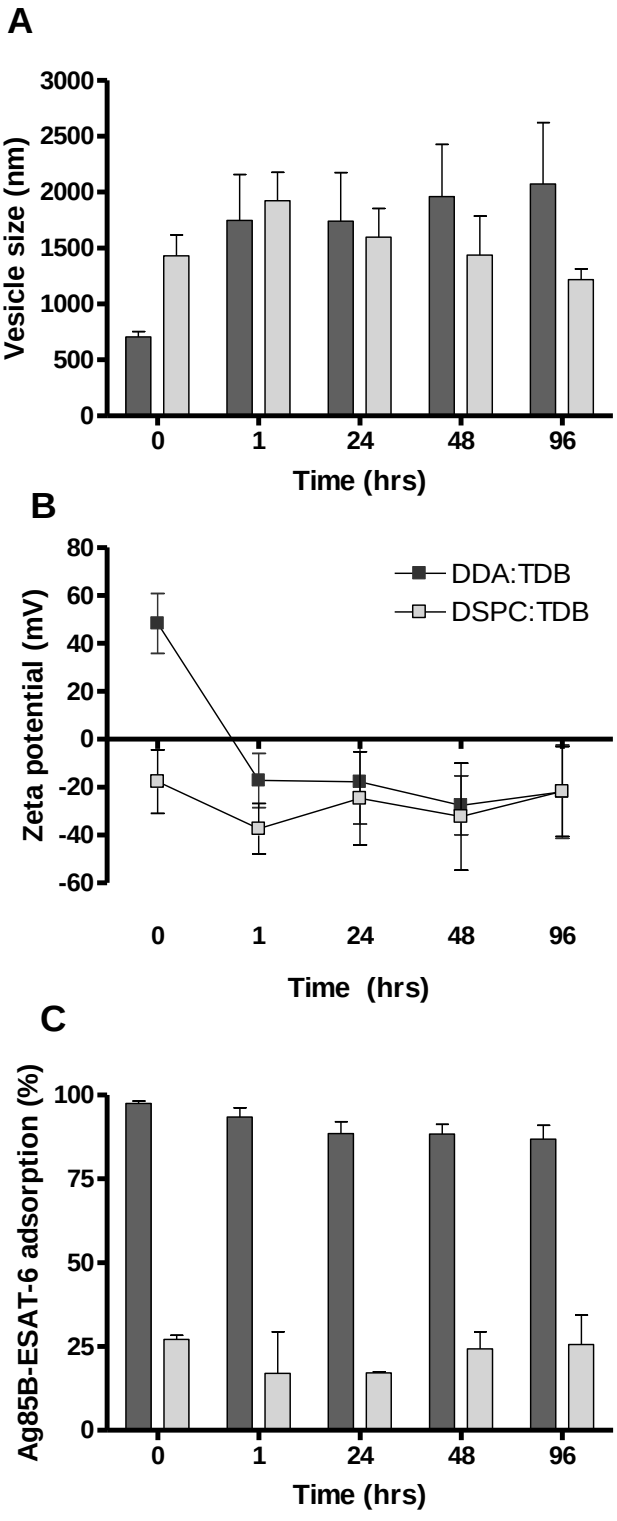

Figure 4.8 Physicochemical characteristics of DDA:TDB and DSPC:TDB liposomes associated with Ag85B-ESAT-6 after placement in a 50 % FCS solution at 37 °C for a 96 hr period. Vesicle size (A) and zeta potential (B) were measured with a Brookhaven ZetaPlus instrument whilst Ag85B-ESAT-6 association to liposomes (C) was measured using ^{125}I-radiolabelled Ag85B-ESAT-6. Results denote mean ± SD of triplicate experiments.

4.3.3 *In vitro* studies of cationic and neutral liposomes

4.3.3.1 Studies using monocyte/macrophage cell lines

Whilst cationic liposomes are commonly associated with toxicity *in vitro* (Felgner et al., 1987; Filion and Phillips, 1997, 1998; Kuo et al., 2005; Lappalainen et al., 1994; Senior et al., 1991), studies have shown that it is not necessarily the same *in vivo* (Hilgers and Snippe, 1992; Larsen et al., 2004), nor is it a standard rule of thumb for all cationic formulations (Filion and Phillips, 1997). DDA-based liposomes were shown to be toxic to monocyte/macrophage cultures at concentrations of ≥ 50 µg/ml (Chapter 3, Figure 3.14, and (Korsholm et al., 2006)). It was therefore investigated whether DSPC:TDB liposomes exhibiting a neutral/anionic surface charge were also toxic to monocyte/macrophage cell cultures at similar concentrations.

The NR and LDH assays were used to measure cell viability and liposome mediated cell toxicity respectively. Unlike DDA based liposomes, DSPC:TDB liposomes were not toxic at any of the concentrations tested (Table 4.2). DDA:TDB and DSPC:TDB liposomes were also added to another macrophage cell line, J774 cells, and phase contrast microscopy used to visualise any liposome-mediated changes in cell physiology. As is seen in Figure 4.9, application of DDA:TDB but not DSPC:TDB liposomes to J774 cells for a 24 hr period led to changes in cell morphology such as cytoplasmic vacuolisation. Vacuolisation is a feature of necrosis, in this case due to cationic-liposome mediated cytotoxicity, and has been observed previously upon exposure of adherent cervical cancer cells to DDA-based liposomes (> 10 µM) (Lappalainen et al., 1994). The cationic liposome-mediated cytotoxicity noted is in contrast to the results presented in Chapter 3 documenting the highest non-toxic concentration of DDA:TDB liposomes to be 5 µg/ml (Figure 3.14).In addition to factors such as differences in cell culture volume (the toxicity assays using 100 µl wells whilst microscopic analysis used 6-well plates), the importance of using multiple assays in parallel to determine cell viability was confirmed, as has been noted by Lappalainen et al. (Lappalainen et al., 1994).

Table 4.2 DSPC:TDB liposome mediated cytotoxic effects on RAW264 cells measured via cell viability ([a], NR assay) and specific cell lysis ([b], LDH assay) assays.

Lipid concentration (µg/ml)	Cell viability (%)[a]	Cell lysis (%)[b]
0.005	84 ± 14	1.4 ± 2.4
0.05	80 ± 20	-0.4 ± 0.6
0.5	87 ± 17	-1.6 ± 3.0
5	86 ± 21	1.9 ± 2.7
50	87 ± 23	1.8 ± 3.0

Results indicate the mean ± SD of three separate experiments in which four wells of a 96-well microplate were assayed per sample. Cell viability was calculated from the % of viable cells without exposure to liposomes and therefore considered 100 % viable. Specific cell lysis is calculated as a % of maximum cell lysis as obtained by Triton® X-100. Lipid concentrations refer to the final DSPC concentration/ml.

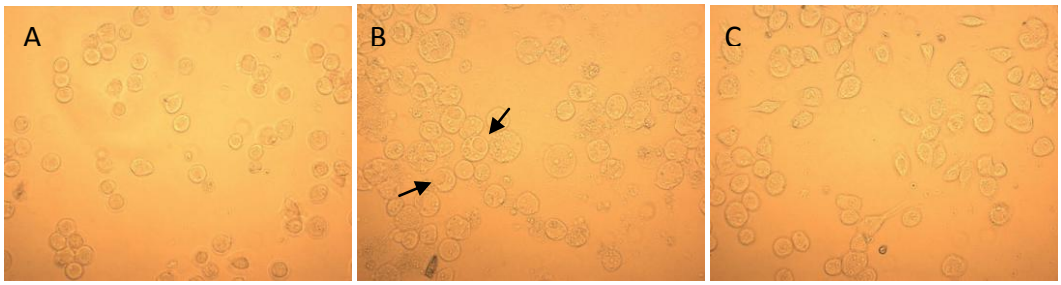

Figure 4.9 Phase contrast images of J774 macrophages without (A) or with DDA:TDB (B) or DSPC:TDB (C) liposomes applied at a final concentration of 5 µg/ml for 24 hrs. Arrows indicate the presence of large internal vacuoles after exposure to DDA:TDB liposomes.

In an attempt to quantify liposome uptake by phagocytes, the human continuous cell line THP-1 treated with vitamin D3 (VD3) was used. THP-1 cells are a monocytic cell line which differentiate into 'alternative' macrophages of a reparative phenotype when stimulated with VD3 for approximately 2 days. The cells are adherent which allows cell monolayers to be effectively washed; when treated with EDTA the cells come into suspension easily. Fluorescently labelled cationic DDA:TDB and neutral DSPC:TDB liposomes were applied to THP-1 cells for up to 4 hrs, following which monolayers were washed to remove liposomes not associated with the cells. As well as applying liposomes to the cells at 37 °C, cells and liposomes were investigated at 4 °C thereby inhibiting endocytosis. The proportion of the total cell population containing or associated with fluorescent liposomes was quantified using flow cytometry.

Figure 4.10 shows the time-dependent uptake of DDA:TDB liposomes after application to THP-1 cells at both 37 °C and 4 °C. No surface stripping of cells to remove adsorbed

liposomes was undertaken and therefore the proportion of liposome +ve cells noted after incubation at 4 °C shows a relatively high proportion of liposomes adsorbing to the cells. In contrast, the application of neutral DSPC:TDB liposomes to cells at either 37 °C or 4 °C resulted in levels of liposome association similar to background fluorescence of the cells (~ 5 %).

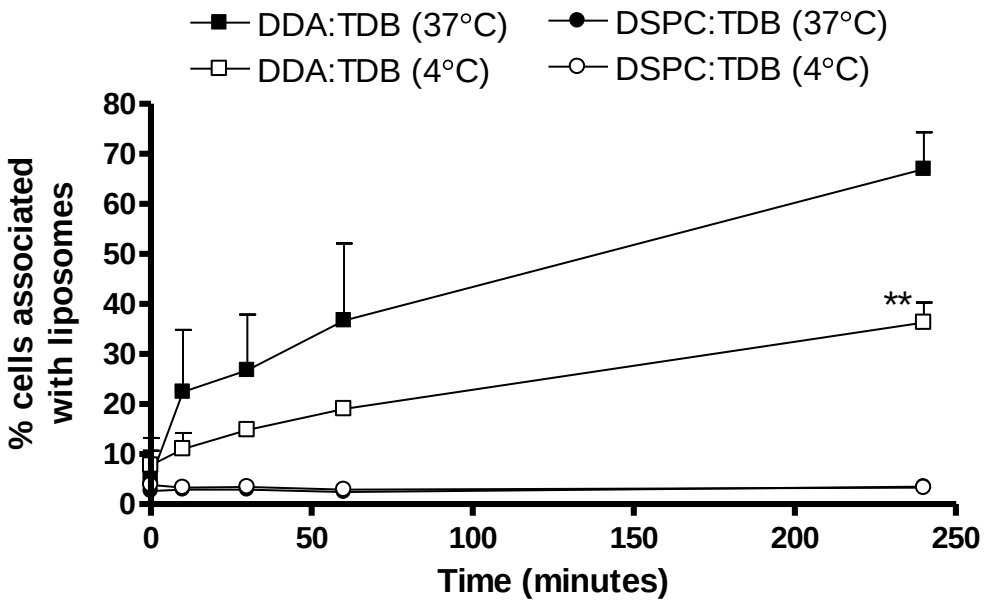

Figure 4.10 Liposome association with THP-1 vitamin D3 stimulated monocytes after incubation at 4 °C or 37 °C for up to 4 hours (240 minutes). **p<0.01 against DDA:TDB liposomes applied at 37 °C.

4.3.3.2 Investigation of cytokine production from dendritic cells

In vitro studies were also conducted to measure the ability of cationic DDA:TDB and anionic/neutral DSPC:TDB liposomes to cause IL-1α and IL-1β production in primary bone-marrow derived DCs (studies undertaken in collaboration with E. McNeela and Dr E. Lavelle, Trinity College Dublin). The IL-1 cytokine family are a group of pro-inflammatory cytokines with widespread actions on the immune system but particularly linked to inflammatory diseases (Dinarello, 1996, 2005; Roux-Lombard, 1998; Sutton et al., 2006). Unlike many other cytokines, IL-1β (like IL-18) production is rather complex as it is produced in an inactive precursor form following activation of Toll-like receptors (TLRs) and activation of the NF-κB pathway (Chapter 1, Figure 1.5). For activation of IL-1β the 'pro' domain must be cleaved and this requires inflammasomes which are themselves composed of an inactive caspase component. Only upon efflux of potassium ions and

binding of ATP to $P2X_7$ receptors does the enzyme dependent activation of caspase 1 occur which can consequently cleave the 'pro' domain from IL-1β (Dinarello, 1996, 2005).

In agreement with previous results obtained from this group where emulsion systems were investigated, application of liposomes without pre-stimulation with LPS did not induce any IL-1α/β production (results not shown). DCs were consequently pre-treated with LPS (5 ng/ml) for 6 hrs followed by application of DDA, DDA:TDB and DSPC:TDB liposomes and measurement of IL-1α and IL-1β in the supernatant after 24 hrs. Cationic DDA and DDA:TDB liposomes were variable in their ability to stimulate IL-1α production by DCs and only after application of the highest concentrations was IL-1α detected (Figure 4.11, A). By contrast, neutral DSPC:TDB liposomes were able to stimulate IL-1α production in a liposome-dose dependent manner (Figure 4.11, A).

IL-1β production was significantly enhanced if DCs were exposed to cationic DDA or DDA:TDB liposomes as opposed to neutral DSPC:TDB liposomes (Figure 4.11, B), and for all liposome formulations a lipid-dose dependent effect was noted. It is interesting to note that the concentrations used were very high compared to the concentrations applied to RAW264 and J774 macrophages; a starting concentration of 500 µg lipid/ml was applied to DCs (1/10 dilution) and only at the 1/640 dilution (equivalent to ~ 8 µg lipid/ml, similar to the highest concentration not found to be toxic to macrophages (Chapter 3, Figure 3.14)) were levels in IL-1α/β non-significant increased compared to LPS-treated cells alone. This suggests that either DCs were more resilient compared to macrophages against the cytotoxic effects of the liposomes, or the liposomes caused cell lysis leading to leakage of IL-1α/β into the supernatant.

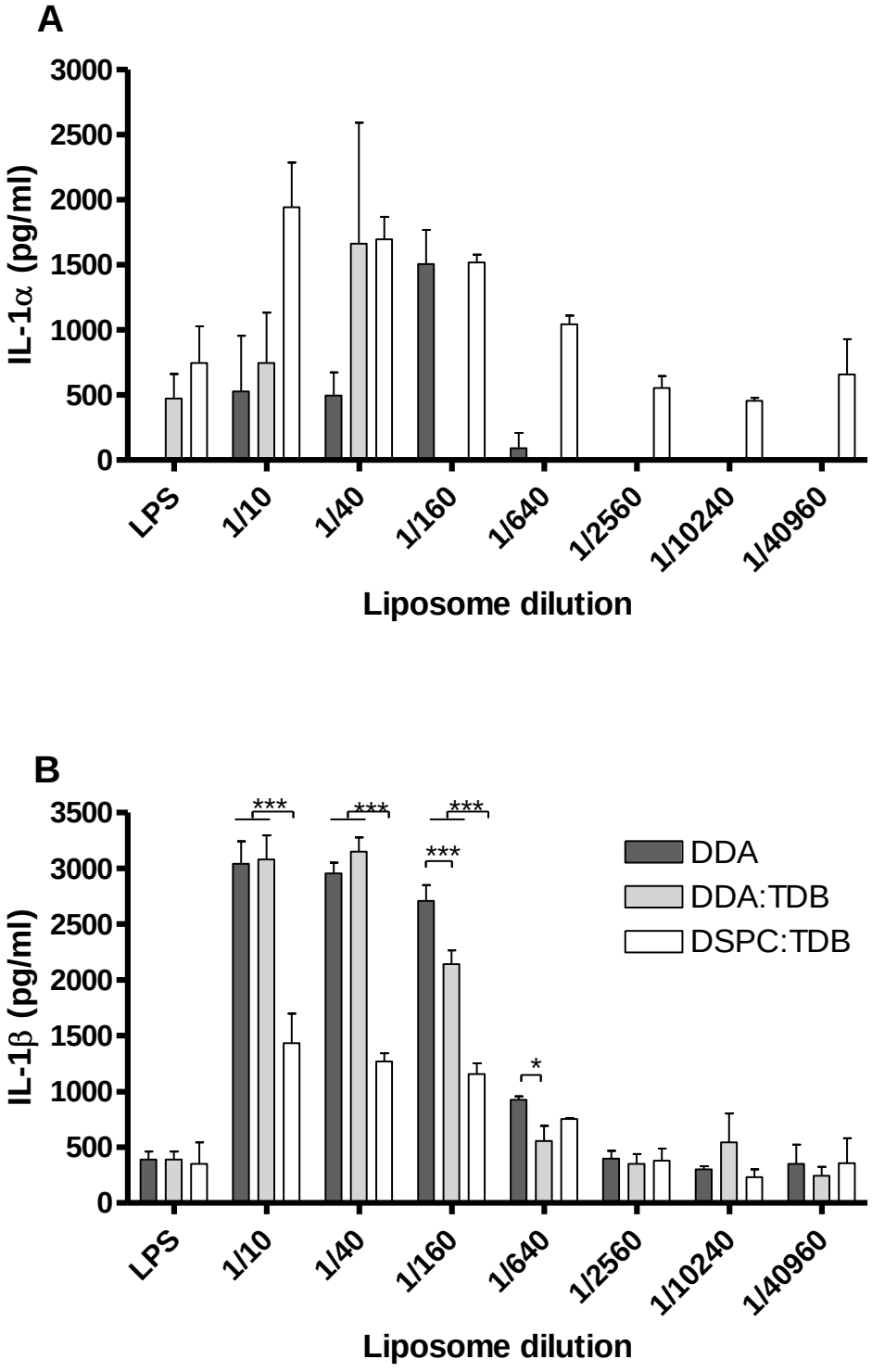

Figure 4.11 IL-1α (A) and IL-1β (B) production from BMDCs stimulated with DDA, DDA:TDB or DSPC:TDB liposomes. Liposomes (5 mg/ml) were applied to DCs starting at a 1/10 dilution and serially diluted thereafter; the supernatant was assayed for the presence of IL-1α and IL-1β after a 24 hr period. All BMDCs were stimulated in the presence of LPS (5 ng/ml). Experiments were conducted at Trinity College Dublin, Ireland. Results denote mean ± SD of triplicate wells. ***, $p<0.001$.

4.3.4 Biodistribution studies: the effect of surface charge and protein adsorption

To determine the effect of liposomes charge on the biodistribution and clearance of liposomes and antigen, a biodistribution study was conducted using the developed dual-radiolabelling protocol (Chapter 3). Both the effect of liposome charge and antigen adsorption on the depot-effect were investigated.

4.3.4.1 Vaccine retention at the SOI

DDA:TDB liposomes showed a similar retention profile at the SOI regardless of whether they were prepared with Ag85B-ESAT-6 or CTH1 antigens (Figure 4.12, A). Between 74 – 84 % of the injected liposome dose was found at the SOI 1 day p.i and this decreased to approximately 30 % of the dose by day 14 p.i irrespective of the antigen used. In contrast, neutral DSPC:TDB liposomes exhibited a faster drainage profile with significantly less ($p<0.001$) liposomes present on days 4 and 14 p.i (Figure 4.12, A). With regards to the movement of antigen from the SOI, two distinct profiles were noted (Figure 4.12, B). Antigen which showed a poor binding ability to liposomes, such as lysozyme with DDA:TDB and Ag85B-ESAT-6 with DSPC:TDB, drained rapidly from the SOI with as little as 7 % of the injected dose detectable 1 day p.i. In contrast, antigen which showed medium/strong adsorption abilities to liposomes, such as noted with CTH1 and Ag85B-ESAT-6 antigen adsorbing to DDA:TDB liposomes, were well retained at the SOI with ~ 55 % of the injected dose present 1 day p.i. On day 4 p.i there were still significant differences noted between the drainage profiles; between 10 – 20 % of injected Ag85B-ESAT-6 and CTH1 was still present whilst the non-adsorbed lysozyme and Ag85B-ESAT-6 administered with DSPC:TDB liposomes was present in very low quantities (~ 3 %). Taken together these results show a clear need for antigen association with liposomes in order for the antigen to be retained at the injection site. Furthermore, prolonged liposome presence at the SOI requires a cationic surface charge which, aside from their role as antigen delivery systems, may be a particularly favourable property for liposomal systems exhibiting immunostimulatory abilities such as the ability to upregulate co-stimulatory molecules or stimulate chemokine production (Vangasseri et al., 2006; Yan et al., 2007). The antigen depot-effect has also been established as an important mechanism of adjuvant action as it allows lengthened antigen exposure to APCs in an environment rich in cellular and molecular immune components (Mosca et al., 2008; Obst et al., 2007).

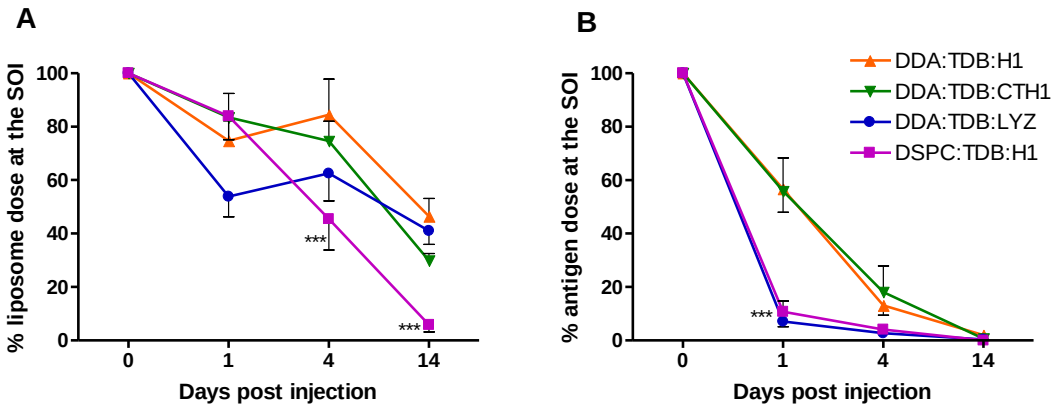

Figure 4.12 Presence of liposome (A) and antigen (B) at the site of injection (SOI) after administration of dual-radiolabelled vaccine formulations. DDA:TDB and DSPC:TDB liposomes were mixed with various antigens (Ag85B-ESAT-6 (H1), CTH1 or lysozyme (LYZ)) and injected via the i.m route. Results denote the mean ± SD of 3 or 4 mice. Significance (***p<0.001) is against DDA:TDB:H1 liposomes at the same time-point.

4.3.4.2 Movement of vaccine components to the local lymph nodes

In addition to investigating the presence of both liposome and antigenic components at the SOI, the local draining lymph nodes (PLN) were removed for vaccine quantification. The results show that faster draining of liposomes to the PLN was linked to liposome charge; DSPC:TDB liposomes drained faster and consequently reached a peak presence on day 4 p.i (Figure 4.13, A). In contrast, cationic DDA:TDB liposomes which exhibited a stronger retention at the SOI drained slower to the PLN. The slower drainage pattern was also dependent on the antigen with which the liposomes were administered; whilst DDA:TDB liposomes adsorbing CTH1 and Ag85B-ESAT-6 (albeit to varying amounts) showed a slow time-dependent increase in liposome presence, the administration of lysozyme with DDA:TDB liposomes (which shows a poor antigen association profile) drained faster and to higher levels. With regards to antigen detection in the PLN, as was seen in Chapter 3, the recovery of antigen in the PLN was low and difficult to quantify (Figure 4.12, B). For all DDA:TDB liposome formulations there were no remarkable differences in the antigen present in the PLN. However, after administration of DSPC:TDB liposomes with Ag85B-ESAT-6 there were elevated levels of Ag85B-ESAT-6 noted on days 1, 4 and 14 p.i. This observation suggests that Ag85B-ESAT-6 passively drains through the PLN when administered with liposomes to which it does not adsorb, such as DSPC:TDB liposomes. In contrast, the same antigen or indeed CTH1 or lysozyme administered with DDA:TDB liposomes does not passively drain through the PLN. However, this raises an

important question: why does lysozyme which does not associate with DDA:TDB liposomes also not show heightened presence in the PLN? One possibility is differences within the antigen itself, as lysozyme is a relatively small protein compared to Ag85B-ESAT-6 (16 kDa vs 44 kDa) and exhibits a high pI of 11 units in contrast to Ag85B-ESAT-6 which has a pI of ~ 4.6 units. It has been suggested that proteins exhibiting extreme pI values may form electrostatic interactions with endothelial cells or components of the extracellular matrix (Melkko et al., 2002). However, this would therefore suggest that lysozyme would be retained at the SOI, although not due to the usual liposome-mediated antigen depot-effect. As lysozyme is not detected at the SOI or in the PLN to particularly high amounts it can only be assumed that lysozyme is removed and degraded, such as occurs naturally with enzymatic degradation of endogenous proteins.

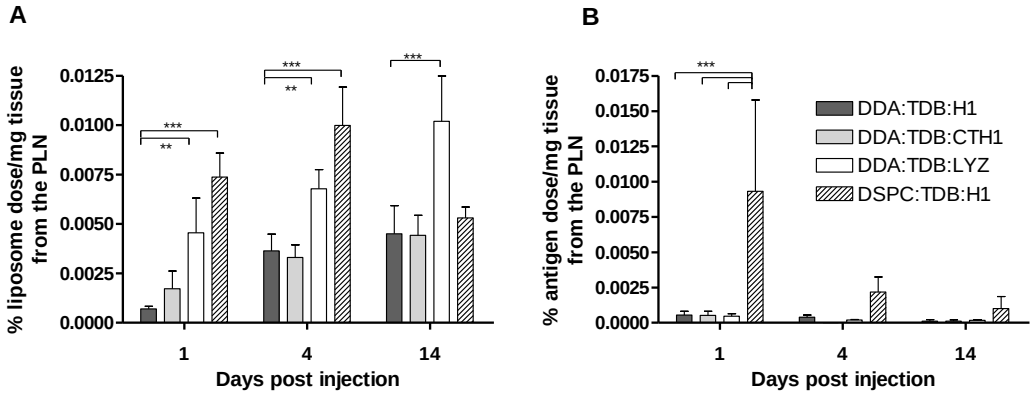

Figure 4.13 Pharmacokinetic profile of liposomes (A) and antigen (B) draining to the local lymph node. DDA:TDB and DSPC:TDB liposomes were mixed with various antigens (Ag85B-ESAT-6 (H1), CTH1 or lysozyme (LYZ)) and injected via the i.m route. Results denote the mean ± SD of 3 or 4 mice. *p<0.05, **p<0.01, ***p<0.001.

4.3.4.3 Use of pontamine blue as a marker for innate immune responses

The slower drainage of DDA:TDB liposomes from the SOI when compared to DSPC:TDB liposomes would suggest that the immune response to the former formulation would be heightened due to potential sustained tissue damage and the presence of inflammatory mediators due to the toxic nature of cationic liposomes (Filion and Phillips, 1997). Immune cells infiltrate to such areas and actively undergo endocytosis which is important for subsequent antigen presentation. In order to determine the level of immune cell influx to the SOI, pontamine blue dye was administered between 4 - 7 days prior to the termination

date. Pontamine blue is known to be readily uptaken by monocytes and therefore offers a simple yet novel method to identify innate immune cell influx in response to vaccine injection.

Figure 4.14 shows tissue from the SOI (quadriceps) collected on days 1, 4 and 14 p.i of DDA:TDB or DSPC:TDB liposomes associated with Ag85B-ESAT-6 antigen. Ag85B-ESAT-6 adsorbing to DDA:TDB liposomes resulted in a strong blue staining at the SOI which increased over time. This is indicative of monocyte influx and is in correlation with data presented in Figures 3.21 and 3.22, Chapter 3. The same strong blue staining was not noted upon administration of DSPC:TDB:Ag85B-ESAT-6 liposomes (Figure 4.14). Although these results are not quantitative, they suggest that cationic liposomes are able to improve phagocyte influx to the site of injection. This is especially important if APC influx is also increased as a result of a liposomal depot-effect, furthermore still if the liposome is able to cause an antigen depot-effect which may therefore promote simultaneous liposome and antigen uptake by APCs.

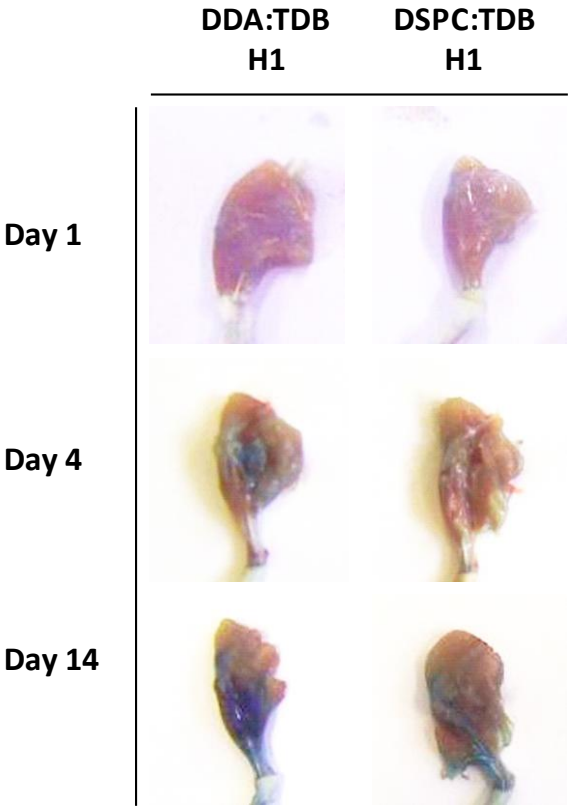

Figure 4.14 Pontamine blue staining at the site of injection (quadriceps muscle) 1, 4 and 14 days post injection (p.i) of DDA:TDB and DSPC:TDB liposomes associated with Ag85B-ESAT-6 (H1).

4.3.5 Ability of liposomes to immunise mice and induce T cell proliferation

In collaboration with Statens Serum Institute (SSI), experiments were conducted to determine the immunising abilities of DDA:TDB and DSPC:TDB liposomes using formulations prepared at Aston University.

4.3.5.1 T cell proliferation measured using antigen-specific transgenic mice

The ability of DDA:TDB and DSPC:TDB liposomes to induce division of T cells expressing a T cell receptor (TCR) for the antigen Ag85B was investigated, thereby determining the antigen presenting abilities of the liposome formulations rather than any cellular activational abilities they may possess. The method used involved fluorescently labelling splenocytes derived from $Ag85B_{241-255}$ TCR transgenic mice with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE), which were then injected into 'normal' C57Bl/6 mice which had been immunised with DDA:TDB or DPSC:TDB liposomes in association with Ag85B-ESAT-6. By measuring the level of CFSE intensity in recipient mice the ability of liposomes to present antigen and induce T cell division could be determined. This assay therefore simulates secondary exposure to antigen and gives an indication as to the intensity of immune response that would be mounted following pathogen infection.

Figure 4.15 shows the % divided cells (A, B) and divisional index (C, D) of cells derived from the lymph nodes of recipient mice either 3 or 14 days after immunisation with the vaccine formulations. All mice exposed to Ag85B-ESAT-6 showed significantly higher levels of T cell division compared to un-immunised control mice (Figure 4.15). Whilst immunisation with Ag85B-ESAT-6 associated DSPC:TDB liposomes did induce division of Ag85B-specific T cells, this was not significantly higher than seen in mice immunised with Ag85B-ESAT-6 alone. By contrast, DDA:TDB liposomes adsorbing Ag85B-ESAT-6 did result in significantly higher ($p<0.05$) levels of T cell division compared to mice immunised with Ag85B-ESAT-6 alone. The increased level of T cell division was deemed significant at both day 3 and 14 p.i, suggestive of long term antigen presentation or stimulation by DDA:TDB liposomes adsorbing Ag85B-ESAT-6.

Figure 4.15 Percentage dividing of- (A, B) and division index for- (C, D) the initial amount of $Ag85B_{241-255}$ specific T cells 4 days after transfer into mice immunized three days (A, C) or 14 days (B, D) previously with Ag85B-ESAT-6 alone (white) or in combination with DSPC:TDB (dashed) or DDA:TDB (grey) liposomes. Taken from (Henriksen-Lacey et al., 2010c).

4.3.5.2 Cytokine production and stage of T cell differentiation

Whilst the results seen in Figure 4.15 show the strong ability of DDA:TDB liposomes to deliver Ag85B-ESAT-6 to APCs for its subsequent presentation to Ag85B specific T cells, the results do not convey the efficiency of DDA:TDB liposomes to induce primary immune responses to antigen. Consequently an immunisation study was conducted whereby C57Bl/6 mice received Ag85B-ESAT-6 alone or in combination with DSPC:TDB or DDA:TDB liposomes. The immune response of mice was measured 3 weeks after the last of 3 immunisations, each given 2 weeks apart. In addition to assessing the cytokine responses of restimulated splenocytes (Figure 4.16, A, B, C), the proportion of $CD44^{High}$ T cells producing IFN-γ, IL-2 and/or TNF-α was determined, thereby giving 8 possible cytokine combinations (Figure 4.16, D).

Figure 4.16 Cytokine production (A, B, C) from restimulated splenocytes derived from mice immunised with Ag85B-ESAT-6 alone or in combination with DSPC:TDB or DDA:TDB liposomes. Detection of cytokines produced from $CD44^{High}$ $CD4^{+}$ T cells (D) was used to determine the stage of T cell differentiation using the model derived by Seder et al. (Seder et al., 2008). Taken from (Henriksen-Lacey et al., 2010c) with minor formatting modifications.

The cytokines secreted emphasise the strong Th1 mediating effect of DDA:TDB liposomes as high levels of IFN-γ (> 20,000 pg/ml) combined with low levels of IL-5 (< 400 pg/ml) were detected from restimulated splenocytes derived from DDA:TDB liposome immunised mice (Figure 4.16, A, B). DSPC:TDB liposomes induced an opposite response characterised by low levels of IFN-γ (not significantly different from Ag85B-ESAT-6 immunisation alone) but with more IL-5. DDA:TDB liposomes were also efficient stimulators of Th17 mediated T cell responses, characterised by the production of IL-17 with levels significantly higher than Ag85B-ESAT-6 immunisation alone or in combination with DSPC:TDB liposomes (Figure 4.16, C). Finally, multiparameter flow cytometry to investigate the stage of T cell differentiation based on cytokine production indicated that DDA:TDB liposomes were efficient stimulators of IFN-γ+TNF-α+IL-2+ multifunctional T cells (~ 31 %) and TNF-α+IL-2+ central memory T cells (~ 36 %), whereas DSPC:TDB liposomes primarily induced TNF-α+IL-2+ (~ 34 %) and TNF-α+ (~ 38 %) central memory T cells (Figure 4.16, D). Based on the progression of T cells from naïve undifferentiated cells towards an IFN-γ producing effector memory cell (Figure 1.4, Chapter 1) (Seder et al., 2008), these results suggest that whilst DDA:TDB liposomes are capable of driving the immune response towards a highly differentiated central memory Th1 cell, DSPC:TDB liposomes lead to only weakly differentiated central memory T cells suggestive of poor abilities to induce long-lived T cell memory responses.

4.4 Discussion and Conclusion

The aim of this chapter was to address the importance of liposome surface charge and liposome association with antigens expressing different pI values and consequently varying abilities to associate with charged vesicles. Understanding such liposome-antigen interactions is of special relevance to subunit protein vaccine design as antigen association with the delivery vehicle (in this case liposomes) may define the immunogenicity of the vaccine.

In Chapter 3 the movement of Ag85B-ESAT-6 from the SOI was investigated; whilst antigen retention at the SOI was far superior after administration with cationic DDA-based liposomes, antigen did drain faster from the SOI than liposomes, suggesting that cationic liposomes become trapped in the tissue. As cationic liposomes interact with serum proteins in an electrostatic manner, the role of charge-elicited liposomal deposition upon *in vivo* administration was investigated. Would neutral liposomes also become 'trapped',

and if not, could the drainage profile of antigens which showed poor adsorption to cationic liposomes also be altered upon injection?

The results obtained suggest that liposome cationic charge is indeed a prerequisite for long term liposome retention at the SOI; liposomes composed of DSPC and therefore expressing a neutral/slightly anionic surface charge drained significantly faster from the injected muscle. In addition it was noted that cationic liposome drainage from the SOI was not related to the antigen with which it was administered as both immunogenic (CTH1 and Ag85B-ESAT-6) and non-immunogenic (lysozyme) antigens injected with cationic DDA:TDB liposomes showed no significant differences in liposome drainage from the SOI. Simultaneously administered antigen did however effect the kinetics of cationic liposome movement to the local draining lymph nodes where initial APC:T cell interactions occur. Among the antigens investigated, three different levels of association with DDA:TDB liposomes were noted. In correlation with Chapter 3 and previous studies (Davidsen et al., 2005), strong adsorption between Ag85B-ESAT-6 and cationic DDA:TDB liposomes was observed. Medium levels of adsorption between CTH1 and DDA:TDB were noted, probably due to the bipolar nature of CTH1 as well as instabilities between the antigen and liposome solutions. Weak association between lysozyme and cationic DDA:TDB liposomes was measured due to the high pI value of lysozyme making electrostatic adsorption unfavourable. Whilst medium/strong levels of antigen adsorption to cationic DDA:TDB liposomes were required for a long term antigen-depot at the SOI, low levels of antigen:liposome association as observed with lysozyme and DDA:TDB liposomes led to rapid removal of antigen.

Neither CTH1 nor lysozyme was investigated in immunisation studies to determine the importance of antigen-liposome association on the ensuing immune responses. However, the role of liposome charge was measured using DDA:TDB (cationic) and DSPC:TDB (neutral) liposomes administered with Ag85B-ESAT-6. The results show a requirement of cationic liposomal charge for the induction of cell mediated immunity characterised by IFN-γ and IL-17 production. Levels of either cytokine were not significantly different to immunisation with Ag85B-ESAT-6 alone when neutral DSPC:TDB liposomes were used as the adjuvant. Furthermore, cationic DDA:TDB liposomes were able to induce multifunctional T cells whilst neutral DSPC:TDB liposomes were not. These findings were measured using multiparameter flow cytometry and give an indication to the abilities of

DDA:TDB liposomes to induce long-term immune responses characterised by memory T cells. Furthermore, DDA:TDB liposomes but not DSPC:TDB liposomes were strong inducers of Ag85B-specific T cell proliferation which was significantly higher than control mice at both early and late time-points.

With regards to the use of DCs as opposed to macrophages, DCs are well known to be the 'professional' antigen presenting cells (APCs) whilst macrophages are known as the 'professional' phagocytic cell. Two of the many excellent in-depth reviews regarding DCs and their antigen presenting abilities include those by Gogolák and colleagues and work from Ralph Steinman's group (Gogolák et al., 2003; Mellman and Steinman, 2001). There is an enormous quantity of research with regards to the specific targeting of vaccine adjuvants to DCs with the aim to improve antigen uptake and presentation on MHC molecules (for a selection see (Boscardin et al., 2006; Broekhoven et al., 2004; Cruz et al., 2010; Sharp et al., 2009; Waeckerle-Men and Groettrup, 2005).

Numerous *in vitro* studies have shown liposome-mediated activation of DCs (Vangasseri et al., 2006; Yan et al., 2007), however, the results obtained are also very dependent on the composition of the cationic liposomes. For example, DDA liposomes do not result in up-regulation of CD40 or CD86 co-stimulatory molecules on DCs (Korsholm et al., 2006), however, when combined with TDB, DDA:TDB liposomes can lead to up-regulation of the same co-stimulatory molecules (CD40 and CD86) suggesting that this attribute is mediated by TDB and not the cationic lipid component (Christensen et al., 2010b; Kamath et al., 2009).

The finding that DDA:TDB liposomes but not DSPC:TDB liposomes induced IL-1β production is of interest for numerous reasons. Firstly IL-1βs production is directly linked to activation of TLRs which include receptors for bacterial substances such as Gram +ve peptidoglycans and lipopeptides (TLRs1/2 and TLRs2/6), flagellin (TLR5), MPL and LPS (TLR4), and numerous synthetic and natural nucleic acids including poly I:C (TLR3), ssRNA (TLR3, 7 and 8), R848 (TLR7 and 8) and CpG motifs (TLR9) (Chapter 1, Figure 1.7). In general, all these TLR-ligand combinations result in the downstream activation of NFκB leading to pro-inflammatory cytokine production and cell proliferation, and also activation of MAP kinases leading to chemokine production and co-stimulatory molecule upregulation. Therefore IL-1β production from cells to which immunomodulatory

liposomes have been applied is directly linked to TLR-liposome interactions and indeed in DDA-based liposomes comprising the TLR4 agonist monophosphoryl lipid A (MPL), IL-1β gene expression was shown to be upregulated after immunisation (Korsholm et al., 2010). Secondly, Il-1β has been shown to promote differentiation of Th17 cells (Sharp et al., 2009) which themselves have an important role in recruitment of IFN-γ producing protective effector cells to the lung after *Mycobacterium tuberculosis* challenge studies (Khader et al., 2007). Importantly, IL-17 production (from Th17 cells) has recently been shown in numerous immunisation studies involving DDA:TDB liposomes adsorbing Ag85B-ESAT-6 (Christensen et al., 2010b; Henriksen-Lacey et al., 2010c; Kamath et al., 2009; Werninghaus et al., 2009).

In this chapter we have shown how DDA:TDB liposomes but not DSPC:TDB liposomes are able to offer a long term antigen depot-effect which results in desirable immune responses. Furthermore, long term exposure to antigen requires a certain degree of cationic liposome-antigen association in order to an antigen-depot effect to ensue.

Chapter 5: Investigating the Choice of Cationic Lipid used within Vaccine Adjuvants

Papers relating to this chapter

Henriksen-Lacey M, Christensen D, Bramwell V.W, Lindenstrøm T, Agger E.M, Andersen P and Perrie Y. Comparison of the depot effect and immunogenicity of liposomes based on DDA, DC-Chol and DOTAP: Prolonged liposome retention mediates stronger Th1 responses. Molecular Pharmaceutics, 2010, *Accepted.*

Christensen D, Henriksen-Lacey M, Kamath A.T, Lindenstrøm T, Korsholm K.S, Christensen J.P, Lambert P.-H, Rochat A, Andersen P, Siegrist C.-A. Perrie Y and Agger E.M. Vaccine adjuvants based on saturated quaternary ammonium lipids have different *in vivo* distribution kinetics and diverse immunological profiles than their unsaturated analogs. *In preparation.*

5.1 Aims

This chapter relates to liposomal adjuvants composed of cationic lipids with documented immunostimulatory actions and comparative membrane fluidity. The physicochemical properties of four cationic liposomes are discussed and their stability assessed in conditions relevant to *in vivo* work as well as shelf-life storage. Cationic liposomes adsorbing tuberculosis antigen Ag85B-ESAT-6 are investigated for their ability to form an antigen depot-effect in dual-radiolabelling biodistribution studies. Furthermore, their ability to induce immune responses is reported as well as collaborative work investigating the ability of liposomes to induce upregulation of co-stimulatory molecules.

5.2 Introduction

In the previous chapters the ability of liposomes to act as an antigen delivery vehicle, in addition to improving immune responses in immunisation studies has been addressed. These results demonstrated the exemplary nature of liposomes composed of dimethyldioctadecylammonium bromide (DDA) in combination with trehalose 6'6-dibehenate (TDB). Termed DDA:TDB liposomes, they are capable of inducing a long term antigen depot-effect, stimulating both the Th1 and Th2 arms of the immune system and have good physicochemical characteristics favouring long term stability (Chapter 3). Substitution of the cationic component DDA with a neutral/slightly anionic lipid led to a poor antigen depot-effect and significantly lower immune responses (Chapter 4). Furthermore, a certain level of antigen adsorption to DDA:TDB liposomes was required for a long-term antigen depot-effect and based on the serum protein induced aggregation noted upon exposure of DDA:TDB liposomes to a simulated *in vivo* environment, it appears that the cationic charge of the liposome plays a key role (Chapter 4). Recently there has been a wealth of interest into the use of cationic structures as adjuvants for subunit protein vaccines. These include immune stimulating complexes exhibiting a cationic charge (PLUSCOMS) (Lendemans et al., 2007; McBurney et al., 2008), cationic peptides such as $KLKL_5KLK$ which is a component of IC-31® adjuvant (Intercell, Austria) (Schellack et al., 2006), cationic bilayer fragments (Lincopan et al., 2009) and most relevantly here, cationic liposomes.

Cationic liposomes have already been extensively investigated as nucleic acid delivery systems due to their ability to complex to, condense and intracellularly deliver anionic nucleic acids such as DNA, mRNA and siRNAs. The first marketed liposome capable of transfecting cells with nucleic material was Lipofectin® (Felgner et al., 1987) which

comprises the cationic lipid 1,2-dioleoyl-distearoyl-3-trimethylammoniumpropane (DOTAP). DOTAP is an unsaturated quaternary ammonium lipid which can form liposomes on its own or with the addition of helper lipids (Simberg et al., 2004). Recently DOTAP liposomes have gained much interest over the last 5 years due to their ability to induce upregulation of co-stimulatory molecules on the surface of dendritic cells (DCs) (Vangasseri et al., 2006). DCs are professional antigen presenting cells (APCs), however, T cell activation can be improved by the upregulation of molecules such as CD80 and CD86. Importantly upregulation, or more precisely their initial production, must be initiated on APCs such as macrophages and B cells as these cells do not constitutively express co-stimulatory molecules which are a requirement for the successful activation of T cells.

Yan and colleagues recently investigated the role of reactive oxygen species (ROS) in the initiation of these activational responses (Yan et al., 2008). They showed that through production of ROS, DOTAP liposomes are able to stimulate the intracellular signalling pathways p38 and ERK which lead to cytokine and chemokine transcription respectively. Furthermore, co-stimulatory molecules CD80 and CD86 are all upregulated upon DOTAP-induced ROS expression (Yan et al., 2008). Among the soluble factors induced by DOTAP liposomes are the chemokines CCL2, CCL3 and CCL4 which are all important monocyte/macrophage inflammatory and chemoattractant substances (Yan et al., 2007). Although liposomes composed solely of DOTAP have not been shown to induce cytokine production in these studies, previous work using liposomes composed of DOTAP, cholesterol, protamine sulphate and plasmid DNA resulted in a Th1 biased cytokine response characterised by IFN-γ, IL-12 and TNF-α production (Whitmore et al., 1999). This is supported by the antagonistic effect DOTAP liposomes have on induction of the Th2 arm of the immune response; both down-regulation of IL-1 signalling and a lack of IL-10 production have been noted (Yan et al., 2007). It therefore appears that DOTAP liposomes are able to initiate a series of intracellular cascades that favour Th1 immune responses characterised by DC activation and recruitment of innate immune cells.

Another cationic lipid which has been implemented to have lipid-specific immunostimulatory actions is N-(N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol), initially described as being a strong mediator of balanced Th1/Th2 responses (Brunel et al., 1999). Brunel and colleagues described the production of cytokines IL-2, IL-5 and IFN-γ, in addition to IgG1 and IgG2 antibody production, in *in vivo* immunisation studies using hepatitis B subunit antigen. In further work using inactivated trivalent split

influenza antigens, the adjuvant effect of DC-Chol liposomes was confirmed and was shown to be dependent on antigen association with the liposomes (Guy et al., 2001). DC-Chol adjuvanted influenza vaccines induced mixed IgG1/IgG2 production with strong hemagglutination inhibition (HI) responses after subcutaneous (s.c) and intranasal (i.n) vaccination (Guy et al., 2001). Studies to elucidate the mechanisms by which DC-Chol liposomes act as adjuvants have suggested a role for the chemokine CCL2, secreted by epithelial cells and involved in Langerhans cell recruitment (Cremel et al., 2006), complement activation (Plank et al., 1996) and the ability of DC-Chol liposomes to associate with antigen and initiate a depot-effect (Guy et al., 2001).

These reports of cationic liposome-mediated immune responses suggest that not only do cationic liposomes initiate their adjuvant abilities by increasing antigen protection and delivery, but also that possible cellular receptors for such lipids (or liposomes) exist. One of the problems in determining whether this is indeed the case is that many of the documented studies use liposomes which are not directly comparable (i.e. due to the use of helper lipids, variable antigens, different administration routes etc). As liposomes composed of DDA:TDB have been shown to induce strong immune responses and an antigen-depot effect, we consequently decided to investigate whether the same was true for liposomes composed of DOTAP or DC-Chol. In order to conduct a direct comparison, liposomes composed of the lipids DDA, DOTAP or DC-Chol in combination with TDB were produced, mixed with the tuberculosis antigen Ag85B-ESAT-6 and administered by the i.m route.

Whilst this selection of liposomes addresses the role of cationic lipid in producing an antigen-depot effect and subsequent immune response, one factor which remains variable is the fluidity of the resulting liposomes. Liposome fluidity is mediated by the main phase transition temperature (T_M) of the lipids, in addition to whether additional components are included in the liposome bilayer. Therefore whilst DDA liposomes express a T_M of 47 °C, addition of TDB (11 mol %) lowers the T_M to 42 °C due to its ability to change the lipid packing efficiency (Davidsen et al., 2005). As the T_M of DDA:TDB liposomes is above 37 °C, upon *in vivo* administration the liposomal bilayers will remain in a rigid state. Whilst the T_M of DOTAP liposomes is not agreed upon (-12 °C, (Hirsch-Lerner and Barenholz, 1998) and ~ 0 °C (Simberg et al., 2004)), it is mutually accepted that it lies below 37 °C due to the presence of unsaturated hydrocarbon tails. Therefore, DOTAP liposome bilayers are fluid at 37 °C upon *in vivo* application. Liposomes composed of DC-Chol are not proposed to

have a specified T_M (as DC-Chol doesn't possess hydrocarbon chains) but the cholesterol planar ring backbone of DC-Chol may indeed reduce bilayer fluidity and in some instances even abolish transition temperatures (Bloom et al., 1991; Gregoriadis and Davis, 1979).

Consequently, it was of interest to investigate an analogue of DDA which exhibited a fluid bilayer upon *in vivo* administration but expressed the same head group and structure so that any receptor mediated liposome:cell interactions would be the same as for DDA liposomes. The lipid N,N-dioleyl-N,N dimethylammonium chloride (DODA) is a structural analogue of DDA with the exception of two unsaturated c=c bonds in the hydrophobic chains. Although little is documented about DODA, its T_M is considered to be < 0 °C (Dennis Christensen, personal communication and (Christensen et al., 2009)) and therefore liposomes composed of DODA are predicted to have a fluid bilayer at 37 °C. Liposomes composed of DDA, DODA, DOTAP or DC-Chol provided an ideal comparison to determine whether changes in biodistribution and/or immune responses are a result of changes in bilayer fluidity (DDA vs DODA vs DOTAP), or changes in the lipid head-group (DDA vs DOTAP vs DC-Chol). The structure of these lipids is shown below in Table 5.1.

Table 5.1 Cationic lipids referred to in Chapter 5

5.3 Results and Discussion

5.3.1 Physicochemical characterisation of cationic liposomes and their ability to adsorb antigens

The lipids DDA, DODA, DOTAP and DC-Chol were all combined in an 8:1 molar ratio with TDB to form liposomes. In correlation with results documented in Chapters 3 and 4, DDA:TDB liposomes were ~ 420 nm in size and expressed a zeta potential of ~ 45 mV (Figure 5.1, A). Upon addition of the TB antigen Ag85B-ESAT-6 (10 µg/ml) no significant changes in vesicle size, zeta potential or polydispersity were noted. Similarly, addition of Ag85B-ESAT-6 to DODA:TDB, DOTAP:TDB or DC-Chol:TDB liposomes did not affect their physicochemical properties at the concentrations used (Figure 5.1, A, B). Whilst DODA:TDB liposomes were of a similar size and surface charge to DDA:TDB liposomes, DOTAP:TDB liposomes were significantly bigger (~ 760 nm) and DC-Chol:TDB liposomes were significantly smaller (~ 220 nm) in size and formed a much more homogenous population (polydispersity of 0.21) compared to DDA:TDB liposomes (Figure 5.1, A). The vesicle size distribution of the liposome populations was confirmed by TEM which showed numerous small vesicles for DC-Chol:TDB liposomes whilst DOTAP:TDB liposomes were much more variable in their structure (Figure 5.2). All liposomes studied expressed a zeta potential of between 40 – 50 mV due to the presence of the ammonium head group conferring a cationic charge (Figure 5.1, B).

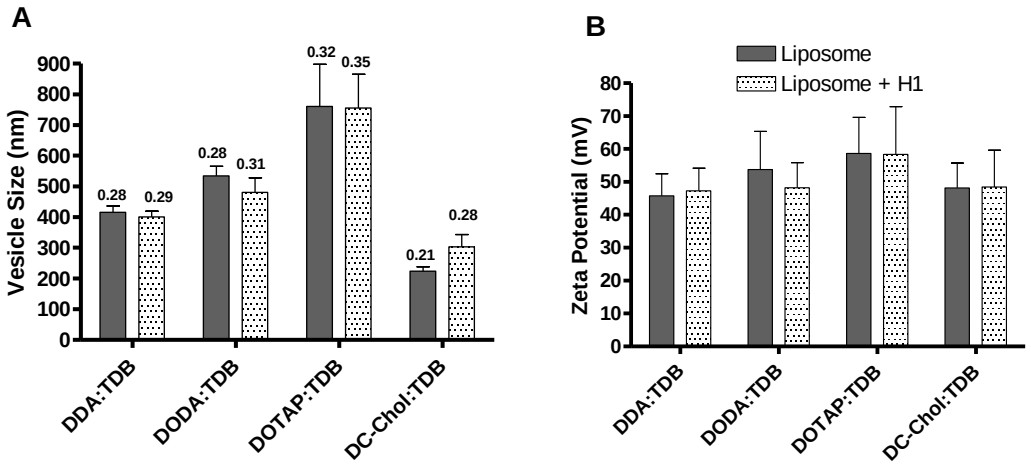

Figure 5.1 Vesicle size (A, bars), polydispersity (A, values) and zeta potential (B) values for liposomes composed of the cationic lipids DDA, DODA, DOTAP and DC-Chol in combination with TDB. Characteristics before (closed bars) and after (open bars) addition of Ag85B-ESAT-6 (10 µg/ml) are shown. Results denote mean ± SD of triplicate samples. H1, Ag85B-ESAT-6.

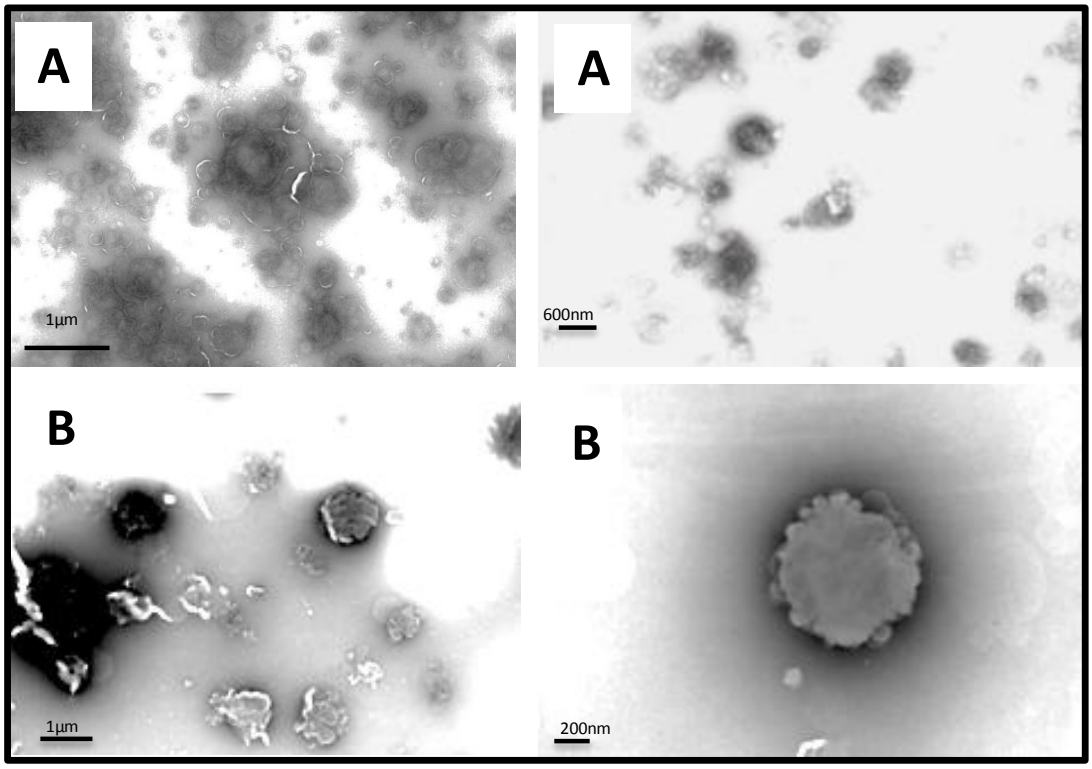

Figure 5.2 TEM images of DC-Chol:TDB (A) and DOTAP:TDB (B) liposomes.

All liposomes were studied over a 56-day period to identify any possible issues with stability. Liposomes were measured for size and zeta potential after storage at both 4 °C and 25 °C. The results obtained suggest that DC-Chol:TDB and DOTAP:TDB liposomes are susceptible to changes in temperature; storage at 4 °C caused a significant increase in the vesicle size of DC-Chol:TDB liposomes as early as 7 days post formation whilst DOTAP:TDB liposomes stored at 25 °C experienced a drop in zeta potential from ~ 55 mV to ~ 10 mV within the initial 10 days (Figure 5.3, C, *d*). The zeta potential of DOTAP:TDB liposomes stored at 25 °C continually decreased over the 56 day period reaching ~ -10 mV by the end of the study. In general, the stability of DDA:TDB and DODA:TDB liposomes was much better with both formulations having a vesicle size of ~ 550 nm after 56 days storage at 4 °C or 25 °C.

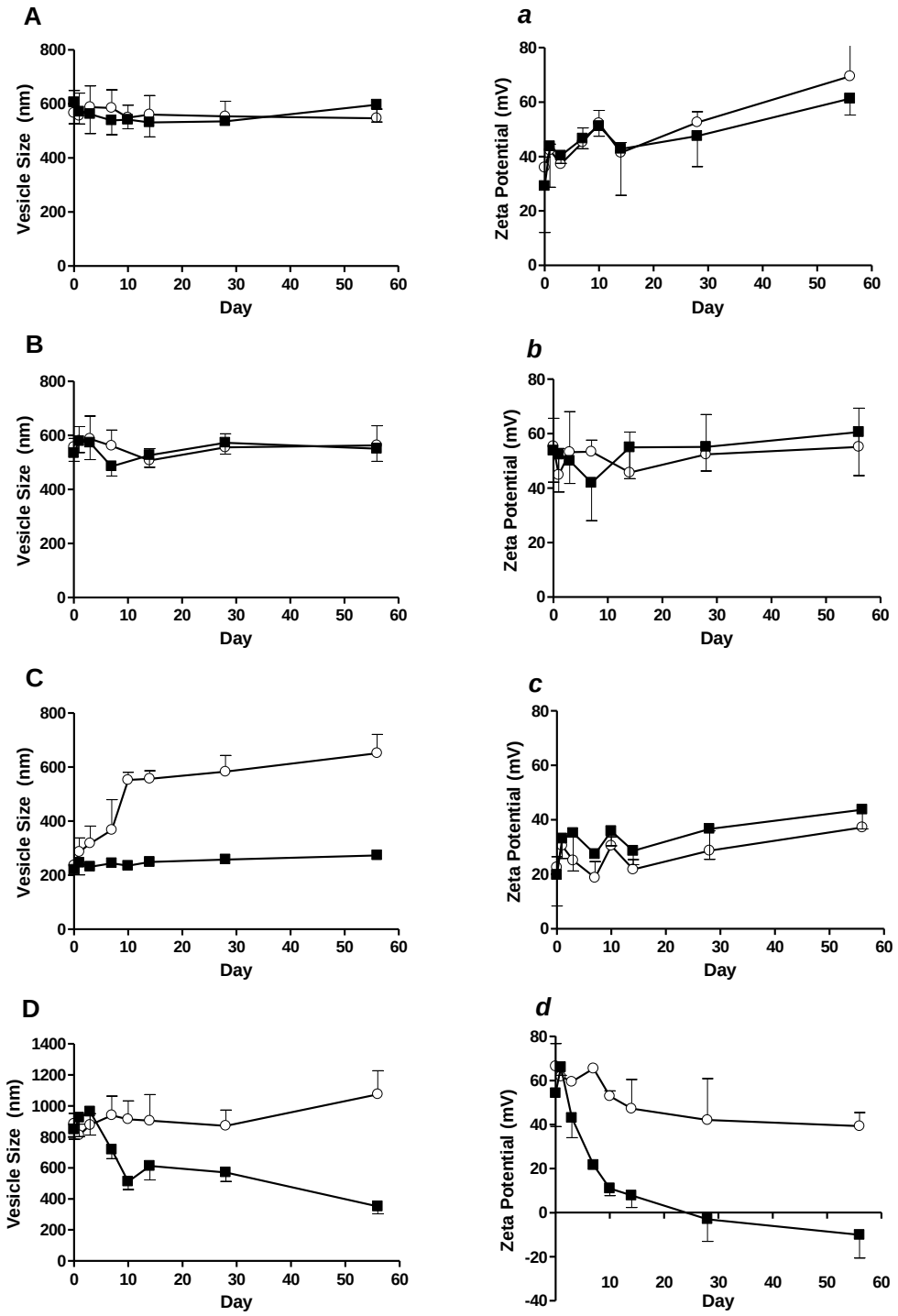

Figure 5.3 Cationic liposomes were investigated for their stability over a 56-day period. The vesicle size (A, B, C, D) and zeta potential (*a, b, c, d*) of liposomes was measured using a Brookhaven ZetaPlus. The liposome formulations included DDA:TDB (A, *a*), DODA:TDB (B, *b*), DC-Chol:TDB (C, *c*) and DOTAP:TDB (D, *d*) which were all stored at 4 °C (○) or 25 °C (■). Results represent mean ± SD of triplicate samples.

To further characterise the liposomal systems, DDA:TDB, DC-Chol:TDB and DOTAP:TDB liposomes were investigated under the same conditions but with the addition of Ag85B-ESAT-6 (10 µg/ml) to the liposomes. Figure 5.4 documents the changes noted in vesicle size and zeta potential; whilst the stability of DC-Chol:TDB liposomes is improved upon addition of Ag85B-ESAT-6, the same temperature dependent effects are noted for DOTAP:TDB liposomes.

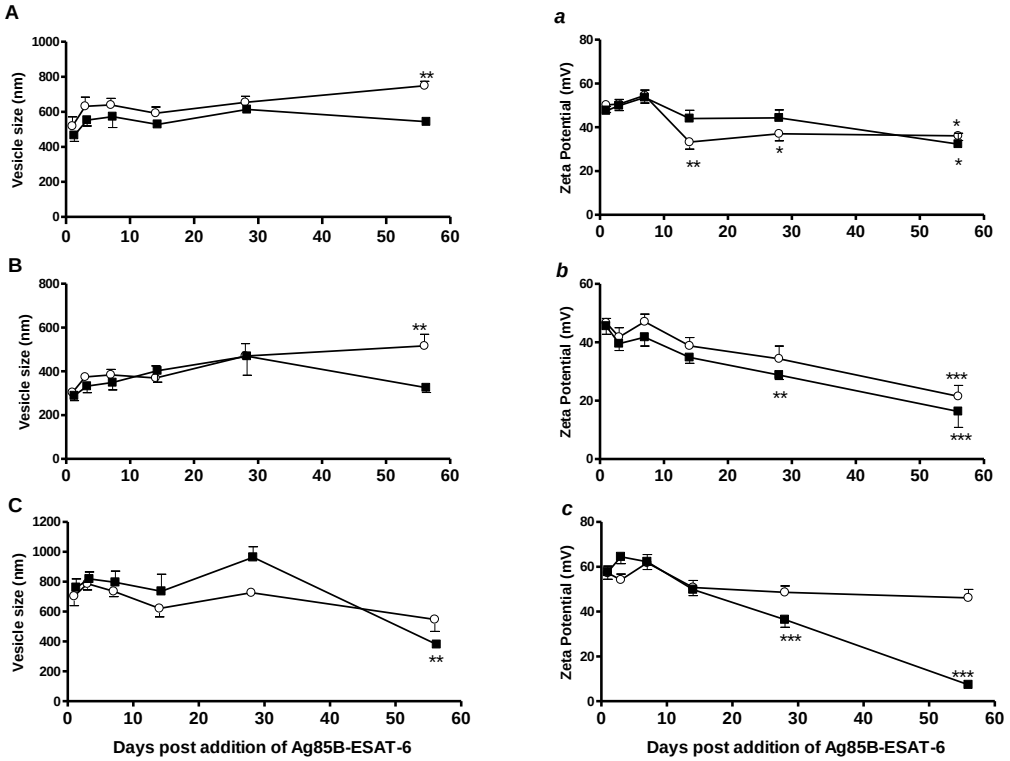

Figure 5.4 Stability of liposomes over a 56-day period as measured by changes in vesicle size (A, B C) and zeta potential (*a, b, c*) after addition of Ag85B-ESAT-6 (10 µg/ml). The liposome formulations DDA:TDB (A, *a*), DC-Chol:TDB (B, *b*) and DOTAP:TDB (C, *c*) were all produced at a final lipid:TDB concentration of 1.98:0.25mM and stored at 4 °C (○) or 25 °C (■). Significant differences in vesicle size and zeta potential over time (compared to day one) are shown. *p<0.05, **p<0.01, ***p<0.001. Results represent mean ± SEM of triplicate samples.

The steep decrease in vesicle size and zeta potential observed after storage of DOTAP:TDB liposomes at 25 °C (Figure 5.3, *d*) could be explained by breakdown of the lipid components. The hydrolysis of lipids containing ester bonds (such as phospholipids and DOTAP) has been reported to affect the physical stability of liposomal systems (Gao and Huang, 1991; Koeber et al., 2007; Rabinovich-Guilatt et al., 2005; Vernooij et al., 2002; Zhong et al., 2010) resulting in a decrease in vesicle size and enhanced permeability of the

bilayer which is also dependent on the T_M of the lipid (Zuidam et al., 1995). Cleavage of the positively charged head-group of DOTAP (Koeber et al., 2007) would result in a decrease in the net surface charge of the liposomes (Figures 5.3 and 5.4) which is known to decrease the stability of the system as the charge repulsion between adjacent liposomes is weaker (Martin, 1990). It is therefore possible that the significant decrease in vesicle size observed for DOTAP:TDB when stored at 25 °C and not at 4 °C is due to hydrolysis of ester linkages and unsaturated carbons present in the hydrocarbon chains which occurs when the liposome exists in its fluid phase.

To further determine how changes in the measured physicochemical properties affect liposome stability, images of DDA:TDB, DC-Chol:TDB and DOTAP:TDB liposomes were taken at the corresponding time-points (Figure 5.5). With the exception of DOTAP:TDB liposomes stored at 25 °C, all liposomes remained stable without any aggregate formation. DOTAP:TDB liposomes aggregated at the latter time-points and clear phase-separation was noted by day 56 (Figure 5.5, insert).

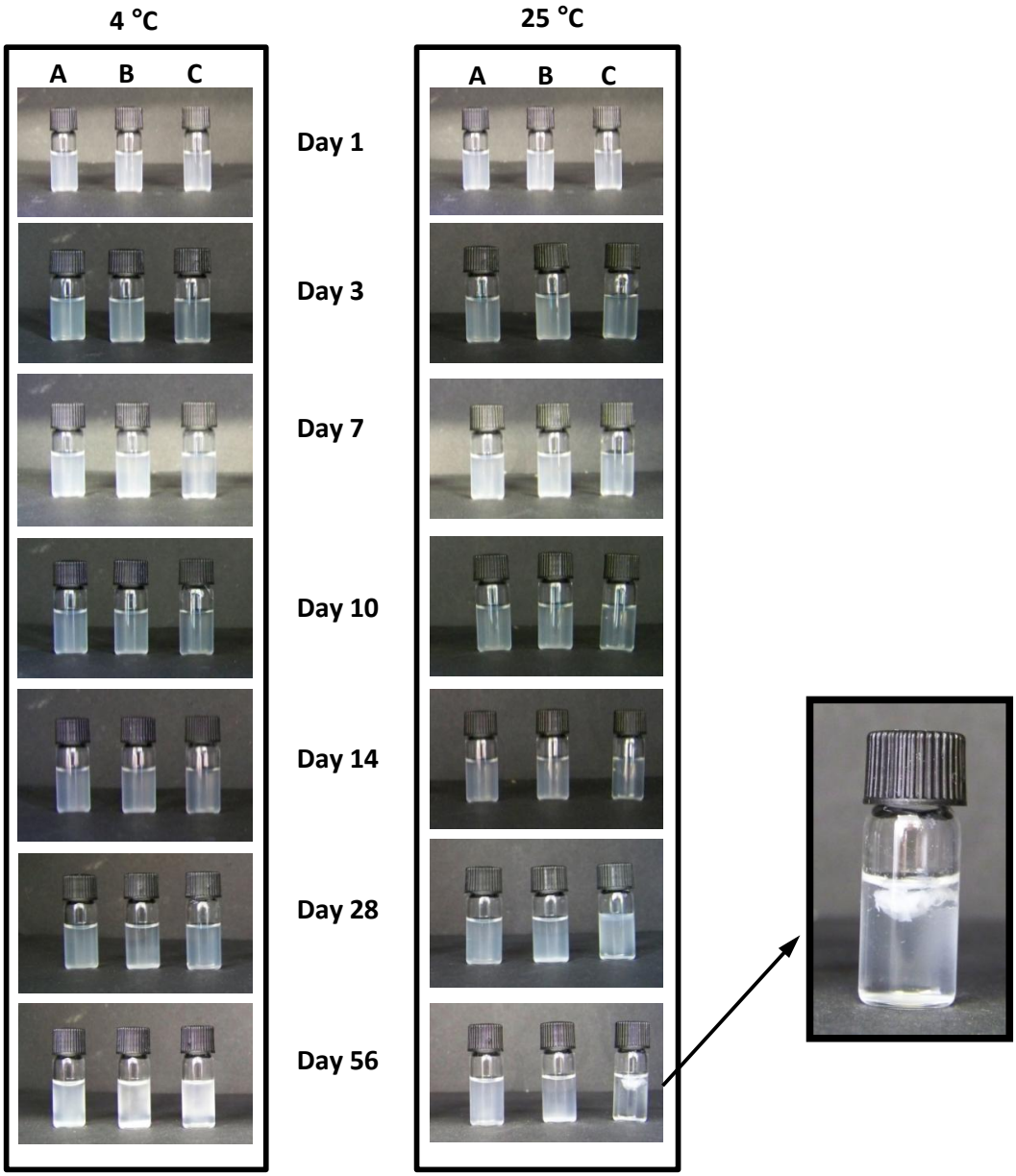

Figure 5.5 Images of DDA:TDB (A), DC-Chol:TDB (B) and DOTAP:TDB (C) liposomes taken at various time-points over a 56-day period. Liposomes were stored at 4 °C or 25 °C. The photo insert shows a closer view of DOTAP:TDB liposomes after storage at 25 °C.

5.3.2 Cationic liposome mediated toxicity studies

Cationic liposomes are significantly more cytotoxic than neutral or anionic liposomes *in vitro* due to the charge mediated interactions between the cell (anionic) and the liposome (cationic) causing high levels of uptake and consequent lysis (Chapter 4). Assuming cytotoxicity is solely mediated by surface charge, it would be expected that more cationic

formulations express higher toxicity whilst lesser cationic formulations are less cytotoxic. The cytotoxicity of DDA:TDB, DODA:TDB, DC-Chol:TDB and DOTAP:TDB liposomes was consequently investigated to test this hypothesis as all 4 formulations expressed non-significantly different zeta potentials and therefore their cytotoxic effects would be expected to be the same. In fact, it was noted that DODA:TDB liposomes were significantly more toxic than DDA:TDB, DC-Chol:TDB and DOTAP:TDB liposomes (Figure 5.6). This was confirmed using both the neutral red (NR) and LDH assays which are complementary assays to determine cell viability and cell lysis respectively. Whilst DDA, DC-Chol and DOTAP based liposomes had no significant effect on cell viability at concentrations < 5 μg/ml, DODA:TDB liposomes induced significant decreases in cell viability at 5 μg/ml. These results therefore suggest that in addition to the surface charge of liposomes having an effect on the cell viability, other factors such as membrane rigidity coupled with vesicle size may play a role. Another more likely possibility is that the toxicity noted with DODA:TDB liposomes is related to the counterion of DODA; whilst DDA has a bromide counterion (Br^-), DODA was supplied with a chlorine counterion (Cl^-). Whilst the effect of counterion has not been studied between DDA and DODA, or indeed using solely DODA, the physicochemical properties of DDA liposomes associated with either Br^- or Cl^- counterions have been studied in detail by Feitosa and colleagues (Feitosa and Alves, 2008; Feitosa et al., 2006; Lopes et al., 2008). Within these studies a larger T_M with more densely packed bilayers was noted for Cl^- counterion DDA liposomes. As no such study has been conducted with DDA (Br^-) and DODA (Cl^-) liposomes, it is difficult to postulate the role of the counterion in the aforementioned cytotoxicity.

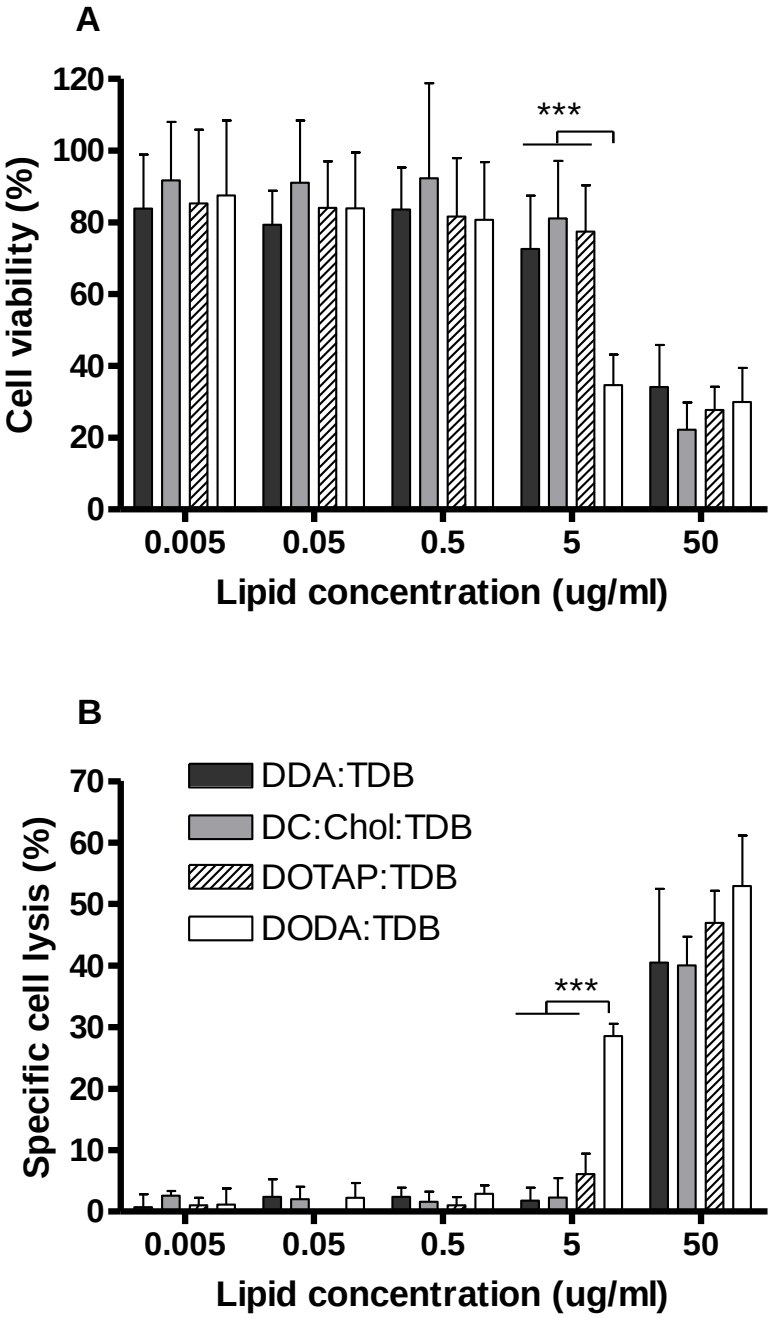

Figure 5.6 Cell viability measured using the neutral red (A) and LDH (B) assays to determine the highest lipid concentration that could be applied to RAW264 cells without significant loss of cell numbers. Results indicate the mean ± SD of three separate experiments in which four wells of a 96-well microplate were assayed per condition. A; cell viability was calculated as a % of cells without any liposomes therefore considered 100 % viable. B; Specific cell lysis is calculated as a % of maximum cell lysis as obtained by Triton® X-100. Two way ANOVA was used to determine significance levels (***, $p< 0.001$).

5.3.3 Studying the biodistribution of cationic liposomal vaccines

5.3.3.1 Ag85B-ESAT-6 adsorption to cationic liposomes

Biodistribution studies were conducted to determine whether cationic lipids expressing different physicochemical characteristics including head group, size and bilayer fluidity affected the strong Ag85B-ESAT-6 antigen depot-effect noted upon its administration with DDA:TDB liposomes (Chapters 3 and 4). The ability of the various cationic lipids to adsorb Ag85B-ESAT-6 was first investigated to ensure that antigen adsorption did indeed occur. As shown in Figure 5.7, all formulations measured high levels of Ag85B-ESAT-6 association ranging between 95 – 97 %. Following this initial adsorption, all formulations were placed in simulated *in vivo* conditions (50 % FCS, 37 °C) and the release profile of Ag85B-ESAT-6 measured. After an initial release period in which DODA:TDB, DC-Chol:TDB and DOTAP:TDB liposomes all showed ~ 10 % loss of Ag85B-ESAT-6, no further significant decreases in Ag85B-ESAT-6 were noted (Figure 5.7). After 96 hours in simulated *in vivo* conditions all liposomes still showed high levels of Ag85B-ESAT-6 adsorption with > 80 % of the initial amount of Ag85B-ESAT-6 added being recovered associated to the cationic liposomes. No significant differences in antigen adsorption to the cationic liposomes were noted (Figure 5.7) suggesting that surface adsorption of Ag85B-ESAT-6 to liposomes expressing a cationic surface charge is indeed due to electrostatic interactions and is not affected by other factors such as bilayer fluidity.

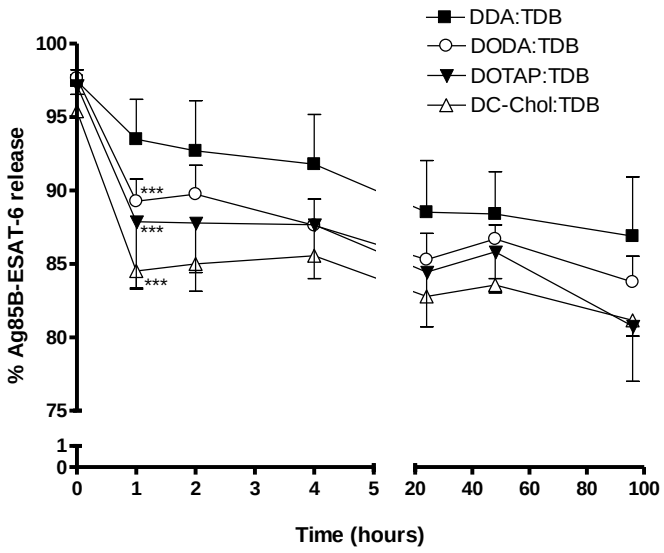

Figure 5.7 Determination of Ag85B-ESAT-6 antigen adsorption to cationic liposomes composed of DDA:TDB, DODA:TDB, DC-Chol:TDB and DOTAP:TDB. Ag85B-ESAT-6 was added at 10 μg/ml and left to adsorb for 1 hr. Liposomes were placed in simulated *in vivo* conditions (50 % FCS, 37 °C) to determine the antigen release profile. ***p<0.001.

5.3.3.2 Determination of vaccine components at the site of injection

Tissue from the site of injection (SOI) was removed on days 1, 4 and 14 p.i and assayed for the presence of ^{3}H (liposome) and ^{125}I (antigen) (Figure 5.8). Two distinct liposome drainage profiles were noted; DDA:TDB and DC-Chol:TDB liposomes exhibited a slower prolonged drainage from the SOI with 30 – 40 % of the injected dose recovered 14 days p.i (Figure 5.8, A). In contrast, DOTAP:TDB and DODA:TDB liposomes drained significantly faster from the SOI and on day 14 p.i between 3 – 10 % of the injected dose was recovered. These differences were deemed significant at all three time-points (Figure 5.8, A). With regards to the retention of Ag85B-ESAT-6 from the SOI, all liposomes were able to initiate a long term antigen depot with over 50 % of the injected dose recovered on day 1 p.i (Figure 5.8, B). By day 14 p.i Ag85B-ESAT-6 could still be detected (between 1 – 7 % of the injected dose).

It was interesting to note that whilst DOTAP:TDB and DODA:TDB liposomes both drained relatively quickly from the SOI, their simultaneously injected Ag85B-ESAT-6 antigen was well retained; this was seen at day 4 p.i where less than 27 % of the injected liposome dose remained in combination with 30 % of the injected antigen dose. This unusual finding whereby the amount of antigen present at the injection site surpassed the amount of liposomes raised the possibility that antigen and liposome dissociation was occurring, although this had not been noted to significantly high levels in the simulated *in vivo* study (Figure 5.7). Furthermore, these results were not due to experimental errors as repeated studies showed the same pattern (results not shown). As only DOTAP:TDB and DODA:TDB liposomes showed this trend, it appears that it is related to bilayer fluidity and it is conceivable that APCs take up antigen at the SOI whereas DOTAP:TDB and DODA:TDB liposomes may either degrade and/or passively drain from the SOI due to the high fluidity of their bilayer at 37 °C. Ultimately there are conditions that occur *in vivo* which have not been replicated in the simulated *in vivo* antigen release study. These include the constant flow of interstitial fluid draining to the lymphatics, tissue infrastructure and the presence of complement able to bind antigens or pathogenic substances. Therefore Ag85B-ESAT-6 association with liposomes *in vivo* may indeed follow a different trend to what is observed in simulated stability studies. A further explanation to the loss of liposome yet retention of antigen at the SOI may be due to loss of the radiolabel itself from the liposomal membrane. ^{3}H-DPPC is added to the liposomes at a low concentration of DPPC (25 ng/dose) yet a high radioactivity so that minimal DPPC interference on the liposomal systems occurs. The critical micellar concentration (CMC) of DPPC is 0.46 mM and

consequently for micelle formation, all of the DPPC added per liposome dose would be required to escape from the liposomal membrane. Therefore whilst solely DPPC micelle formation seems unlikely, another possibility is that DPPC fuses into the cell membrane. However if this were the case, ^{3}H-DPPC would be recovered from the SOI to higher amounts and for a longer period of time than is noted. A further possibility would be the loss of ^{3}H itself from DPPC however dialysis of liposomes containing ^{3}H-DPPC in conditions simulating the *in vivo* environment (50 % FCS, 37 °C) did not show any loss of radioactivity (Chapter 3, Figure 3.11). With all this in mind, it would appear that the decrease in liposomes draining from the SOI whilst antigen is retained is due to antigen dissociation from the liposomes and different drainage profiles.

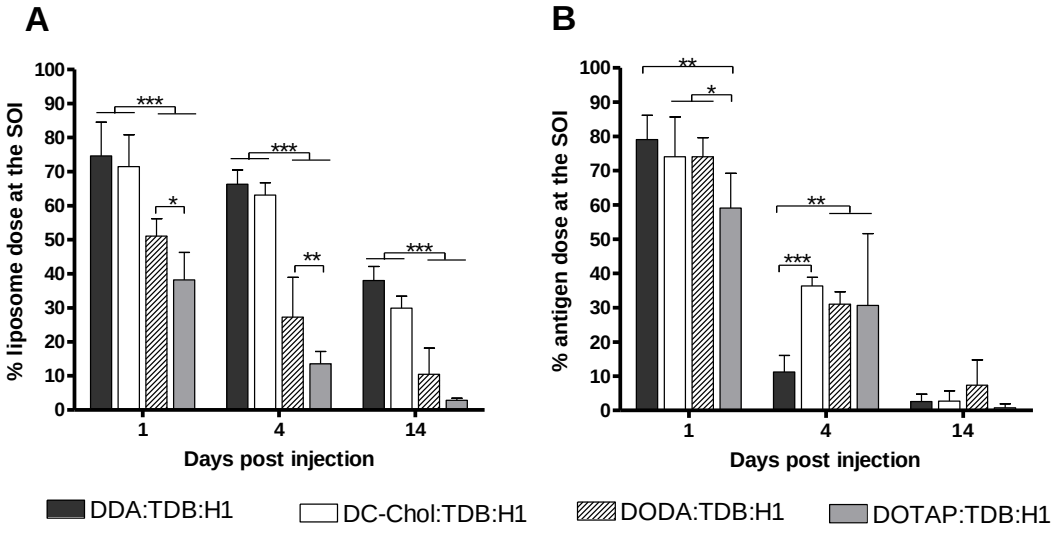

Figure 5.8 Biodistribution studies in which tissue from the site of injection (SOI) was removed and the presence of liposomes (A) and protein (B) determined. Liposomes investigated include DDA:TDB, DC-Chol:TDB, DODA:TDB and DOTAP:TDB, all associated with Ag85B-ESAT-6 antigen. Results denoted mean ± SD of 5 mice. H1, Ag85B-ESAT-6. *p<0.05, **p<0.01, ***p<0.001

5.3.3.3 Determination of vaccine components in the local draining lymph node

The popliteal lymph node (PLN) was removed to assess the proportion of liposome and antigen (Figure 5.9, A and B respectively) that had drained from the SOI to the local lymphoid organ. In a similar way to the results relating to vaccine presence at the SOI (Figure 5.8), there were 2 distinct patterns of liposome drainage to the PLN. DDA:TDB and DC-Chol:TDB liposomes showed a gradual increase in liposome presence which by day 14

continued to rise. In contrast, the movement of DODA:TDB or DOTAP:TDB liposomes to the SOI showed a peak in liposome presence at day 4 p.i, after which the levels dropped to below that noted on day 1 p.i. Injection of DOTAP:TDB liposomes led to the highest recovered liposome dose on days 1 and 4 p.i whereby between 0.008 – 0.01 % of the injected dose per mg of tissue was detected. Similar levels of liposome presence were noted for DDA:TDB liposomes by day 14 p.i, suggestive of a much slower drainage pattern but with equal amplitude. By day 14 p.i the presence of DDA:TDB liposomes was significantly higher ($p<0.001$) than that of DC-Chol:TDB, DODA:TDB or DOTAP:TDB liposomes (Figure 5.9, A).

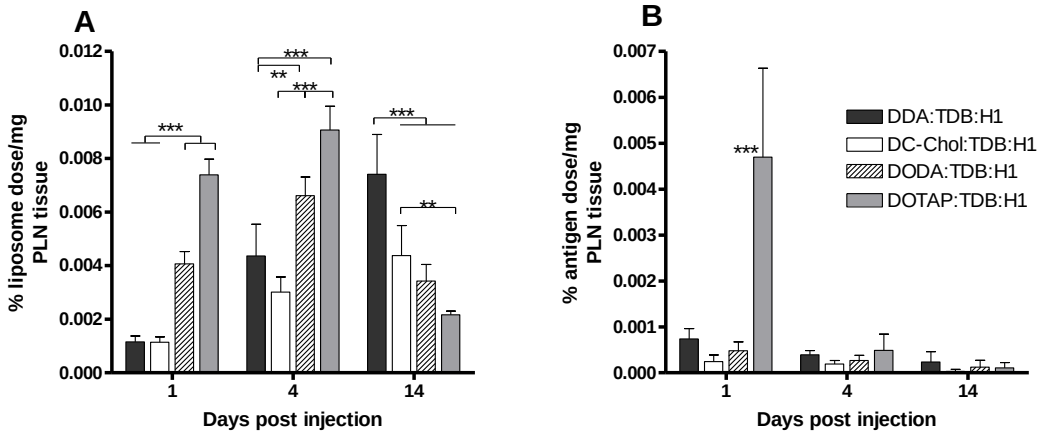

Figure 5.9 Liposome (A) and Ag85B-ESAT-6 antigen (B) drainage kinetics from the SOI to the local draining popliteal lymph node (PLN), detected on days 1, 4 and 14 p.i. Results denote mean ± SD of 5 mice. H1, Ag85B-ESAT-6. **p<0.01, ***p<0.001

In correlation with previous results documenting antigen presence in the PLN (Chapter 3 and 4), the proportion of the injected dose was very low and difficult to compare between liposomal formulations. One notable exception to this was the administration of Ag85B-ESAT-6 with DOTAP:TDB liposomes whereby significantly higher ($p<0.001$) amounts of Ag85B-ESAT-6 were detected on day 1 p.i (Figure 5.9, B). A similar antigen drainage profile has also been noted for DSPC:TDB liposomes associated with Ag85B-ESAT-6 (Chapter 4, Figure 4.12). The results are unusual as both DOTAP:TDB liposomes and DSPC:TDB liposomes also show similar liposomal drainage kinetics to the PLN even though their physicochemical characteristics are rather different. There is a possibility that the physicochemical characteristics of DOTAP:TDB liposomes change *in vivo* and it may be that the temperature affects the ester bonds resulting in hydrolysis and subsequent cleavage

of the positively charged head-group of DOTAP (Koeber et al., 2007). If this did occur, not only would the bilayer stability of DOTAP:TDB liposomes be severely altered but antigen dissociation would occur allowing free antigen to passively drain through the PLN as seen with non-Ag85B-ESAT-6 adsorbing DPSC:TDB liposomes (Chapter 4). Whilst this theory may indeed explain the results seen *in vivo*, unfortunately serum stability experiments designed to simulate the *in vivo* environment did not support this hypothesis as Ag85B-ESAT-6 adsorption to DOTAP:TDB liposomes was found to be similar to the other cationic liposome formulations tested (Figure 5.7).

5.3.3.4 Monocyte recruitment to the SOI

In previous chapters the use of the dye pontamine blue has allowed monocyte influx to the SOI to be semi-quantified based on the intensity of blue staining recovered. Pontamine blue is readily uptaken by macrophages (Tilney, 1971) and can therefore give an indication to the ability of liposomes to induce innate immune cell influx to the injected tissue. As noted in Figure 5.10, injection of DDA:TDB, DC-Chol:TDB or DOTAP:TDB liposomes, all adsorbing Ag85B-ESAT-6, does not lead to high levels of blue staining on day 1 p.i. However, by day 4 p.i more significant blue staining was noted after injection of DDA:TDB and DOTAP:TDB liposomes whilst DC-Chol:TDB injection induced little pontamine blue staining. By day 14 p.i the staining intensity increased for DDA:TDB and DC-Chol:TDB liposomes whilst a slight decrease was noted after DOTAP:TDB liposome injection. These results correlate with the kinetics of liposome drainage to the PLN (Figure 5.9) and suggest a slower ability of DC-Chol:TDB liposomes to initiate monocyte influx to the SOI. Whilst the presence of pontamine blue cannot be quantified (as the tissue is radioactive and is required for quantification of the liposome and antigen dose at the SOI), the images in Figure 5.10 give a good indication as to the immunostimulatory abilities of the different liposome formulations.

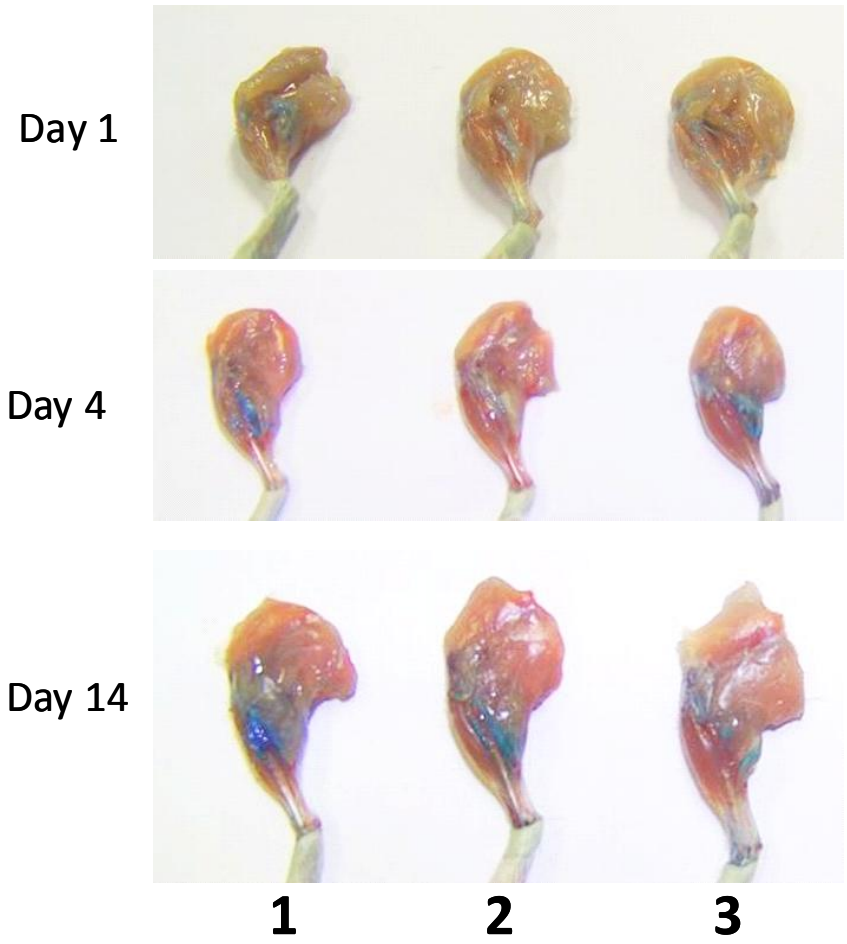

Figure 5.10 Pontamine blue staining at the site of injection (quadriceps) after injection (i.m) of DDA:TDB (1), DC-Chol:TDB (2) or DOTAP:TDB (3) liposomes, all adsorbing Ag85B-ESAT-6 antigen.

5.3.4 Immunogenicity of cationic liposomes adsorbing Ag85B-ESAT-6 antigen

In addition to conducting biodistribution studies to identify the ability of cationic liposomes to induce a long term antigen depot-effect, the immune responses to the various cationic formulations including T cell proliferative responses and cytokine production were measured in C57Bl/6 mice. These data describe results obtained from experiments conducted in collaboration with Statens Serum Institute (SSI) using liposomes made at Aston University.

5.3.4.1 T cell division in response to cationic liposomal vaccines

In order to determine the efficiency of Ag85B-ESAT-6 adsorbing cationic liposomes to cause T cell division, splenocytes derived from $Ag85B_{241-255}$ TCR transgenic mice were

fluorescently labelled with CFSE and injected into C57Bl/6 mice previously immunised with DDA:TDB, DC-Chol:TDB or DOTAP:TDB liposomes. Determination of the decrease in CFSE per cell gives an indication as to the level of cellular division (or proliferation) in response to Ag85B-ESAT-6; this method is described in more detail in Chapter 4 (Section 4.3.5.1). Injection of Ag85B-ESAT-6 alone was able to induce T cell division to significantly higher ($p<0.01$) levels than naïve unimmunised control mice (Figure 5.11, A, C). This however was only noted in the early stages of Ag85B-ESAT-6 exposure as at day 14 no significant increase in T cell division above levels of unimmunised mice was noted after immunisation with Ag85B-ESAT-6 alone. As expected, DDA:TDB, DC-Chol:TDB and DOTAP:TDB liposomes were also able to induce significantly higher levels of T cell division compared to naïve mice, however, significantly higher levels compared to immunisation with Ag85B-ESAT-6 alone was only noted for DDA:TDB liposomes (Figure 5.11, A, C). Extended proliferative responses measured on day 14 p.i showed that only DOTAP:TDB and DDA:TDB liposomes were capable of causing significant increases in T cell division, measured by the % divided cells as opposed to the divisional index (Figure 5.11, B vs D).

Comparison of the three cationic liposome formulations with each other suggests an equal ability of the liposomes to present Ag85B-ESAT-6 to APCs as no significant differences between liposome formulations were noted. However, whilst the results indicate that all three formulations were able to present antigen at the early time-points, the response was short-lived as by 14 days p.i no significant differences against mice immunised with only Ag85B-ESAT-6 were noted. These results demonstrate antigen presenting abilities for DDA, DC-Chol and DOTAP:TDB liposomes, however those attributed to DDA:TDB liposomes are superior in the initial stages of immunisation. Interestingly no particular trend with regards to liposome membrane rigidity is noted which is in contrast to the movement of liposome from the SOI (Figure 5.9, A).

Figure 5.11 Percentage dividing of- (A, B) and division index for- (C, D) the initial amount of $Ag85B_{241-255}$ specific T cells 4 days after transfer into mice immunized three days (A, C) or 14 days (B, D) previously with Ag85B-ESAT-6 alone (white) or in combination with DOTAP:TDB (black), DC-Chol:TDB (dashed) or DDA:TDB (grey) liposomes. Taken from (Henriksen-Lacey et al., 2010b).

5.3.4.2 Cationic liposomal vaccine induced cytokine responses

The ability of cationic liposomes to induce a primarily Th1 or Th2 response was investigated by measuring IFN-γ (Th1) and IL-5 (Th2) production from peripheral blood mononuclear cells (PMBCs) of immunised mice. Samples were collected on day 7 and 21 post the final injection (p.f.i) of triplicate immunisations with 2 week intervals. Immunisation with Ag85B-ESAT-6 alone did not induce any IFN-γ production and levels of IL-5 were low, albeit deemed significantly different on day 21 p.f.i (Figure 5.12, D). DOTAP:TDB liposomes were unable to stimulate significantly elevated levels of IFN-γ production on day 7 or 21 p.f.i compared to naïve control or Ag85B-ESAT-6 immunised mice (Figure 5.12 1, A, C). In contrast, DC-Chol:TDB liposomes were able to stimulate IFN-γ production but only at the early time-point as by day 21 p.f.i IFN-γ production was not deemed significantly higher than control or Ag85B-ESAT-6 immunised mice. All formulations were capable of IL-5 production and whilst low (< 8 pg/ml), this was significantly higher than naïve or Ag85B-ESAT-6 immunised mice at both day 7 and day 21 p.f.i; no significant differences were noted between cationic liposome formulations. DDA:TDB liposomes were consequently the only cationic liposomal formulation able to stimulate IFN-γ production to relatively high levels over a continued period of time (Figure 5.12, A, C).

Whilst cationic charge and membrane rigidity play an important role in liposome retention at the SOI (Figure 5.8, A), the cytokine results suggest that there is another parameter which affects the ability of cationic liposomal adjuvants to stimulate long term T cell memory responses, such as that noted after immunisation with DDA:TDB liposomes. DC-Chol:TDB liposomes, although able to stimulate immune responses, are poor inducers of long term memory T cells as highlighted by the disappearance of IFN-γ responses 21 days after the last immunisation. However results relating to liposome retention at the SOI, as well as the continued ability of DC-Chol:TDB immunised mice to produce IL-5 production, suggests that this formulation does indeed have immunostimulatory abilities due to a combination of prolonged presence at the SOI and an ability to improve Ag85B-ESAT-6 presentation to T cells.

Figure 5.12 IFN-γ (A, C) and IL-5 (B, D) responses in mice (pooled PBMCs) one (A, B) and three (C, D) weeks after the last of three immunizations with 2 µg of Ag85B-ESAT-6 antigen alone (white) or combined with DOTAP:TDB (black), DC-Chol:TDB (dashed) or DDA:TDB (grey) liposomes. Results denote mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. Taken from (Henriksen-Lacey et al., 2010b) with minor formatting modifications.

In a separate experiment, the ability of DODA:TDB liposomes to stimulate cytokine production from PMBCs was investigated. In addition to analysing IFN-γ and IL-5 production, characteristic of Th1 and Th2 immune responses respectively, IL-17 production was also measured as restimulated splenocytes derived from DDA:TDB liposome immunised mice were previously shown to induce IL-17 production (Chapter 4, Figure 4.16). Both DDA:TDB and DODA:TDB liposomes were able to induce IFN-γ, IL-17 and IL-5 7 days p.f.i (Figure 5.13). Absent IFN-γ and IL-17 responses were noted for Ag85B-ESAT-6 immunised mice. In correlation with Figure 5.12, DDA:TDB liposomes immunised mice exhibited a decline in IFN-γ but an increase in IL-5 production with time (day 7 vs day 21 p.f.i). Remarkably considering their structural similarity, DODA:TDB liposomes failed to induce IFN-γ to even remotely similar levels as DDA:TDB liposomes; whilst the same pattern was noted for DODA:TDB liposomes, the amplitude of their response was

significantly lower with IFN-γ production being ~ 100-fold less on day 7 p.f.i. (Figure 5.13, A).

Figure 5.13 Cytokine responses from PMBCs derived from mice immunised with Ag85B-ESAT-6 alone or in combination with DODA:TDB or DDA:TDB liposomes. Mice received 3 immunisations with 2 week intervals. Samples were collected on day 7 or 21 post the final immunisation and the mean ± SEM is shown. *p<0.05, **p<0.01, ***p<0.001. Taken from (Christensen et al., 2010b) with minor formatting modifications.

T cells derived from mice immunised with Ag85B-ESAT-6 combined with DODA:TDB and DDA:TDB liposomes were also analysed by multiparameter flow cytometry to investigate the stage of T cell differentiation. DDA:TDB liposomes induced predominantly multifunctional T cells being IFN-γ+TNF-α+IL-2+ (~ 30 %) and central memory T cells being TNF-α+IL-2+ (~ 32 %) (Figure 5.14). DODA:TDB liposomes primarily induced TNF-α+IL-2+ (~ 42 %) and TNF-α+ (~ 28 %) central memory T cells, albeit to lower levels compared to the response noted from DDA:TDB immunised mice. The phenotype of T cells derived from DODA:TDB liposome immunised mice is remarkably similar to that derived from DSPC:TDB liposome immunised mice (Chapter 4, Figure 4.16, D) and suggests an inability of DODA:TDB liposomes to induce memory T cell production.

Figure 5.14 Detection of cytokines produced from $CD44^{High}$ $CD4^{+}$ T cells was used to determine the stage of T cell differentiation using the model derived by Seder et al. (Seder et al., 2008). Results denote mean ± SEM. Taken from (Christensen et al., 2010b) with minor formatting modifications.

5.4 Discussion and Conclusions

The results shown in this chapter aimed to clarify the role of the lipid DDA in the positive immune responses which have been noted in numerous immunisation studies using the tuberculosis antigen Ag85B-ESAT-6. Liposomes composed of DDA combined with the immunomodulator TDB (DDA:TDB liposomes) form a strong liposome and antigen depot-effect at the SOI, elicit Th1 cell-mediated immune responses characterised by IFN-γ and IL-17 production, induce multifunctional and memory T cells, effectively present antigen to T cells (as measured by their proliferative ability upon subsequent exposure to antigen), and are also capable of inducing humoral immunity with high IgG1 and IgG2 titres. Although cationic charge has been noted as an important factor for the formation of the above responses (Chapter 4), it is unclear whether many of the observed effects are as a result of the lipid DDA itself, or possibly a synergistic combination between cationic lipid and TDB. By investigating the depot-forming and immunising abilities of other similar cationic liposomes as adjuvants it was hoped to gain a more detailed understanding of the mechanisms by which cationic liposomes act as adjuvants.

Based on differences in membrane fluidity (defined by the inclusion of unsaturated hydrocarbon chains in the lipids) and previously documented immunostimulatory actions, three cationic lipids were chosen to form comparative cationic liposomes to DDA:TDB. All the cationic liposome formulations adsorbed TB antigen Ag85B-ESAT-6 to high levels (> 95 %) due to the electrostatic interaction of Ag85B-ESAT-6 with the highly positive surface of the liposomes. Upon i.m injection of the cationic liposomal vaccines, two distinct profiles of liposome movement from the SOI were noted; liposomes possessing a rigid bilayer (DDA:TDB and DC-Chol:TDB) showed a lengthened retention profile whilst those with a fluid bilayer (DOTAP:TDB and DODA:TDB) drained significantly faster from the SOI. Liposome rigidity correlated with IFN-γ production at the early stages of immunisation (1 week after the final injection), however, 3 weeks after the final injection IFN-γ production from rigid DC-Chol:TDB liposome immunised mice had waned whilst DDA:TDB liposome immunised mice retained the ability to produce IFN-γ. All cationic liposomes with documented immunostimulatory action (DDA-, DC-Chol- and DOTAP-based liposomes) were able to induce similar levels of IL-5 which were significantly higher than after immunisation with Ag85B-ESAT-6 alone. Liposomes composed of the lipid DODA, chosen for its fluid membrane structure as opposed to immunisation abilities, were also able to stimulate IL-5 production, However, the response took longer to establish with significant

levels only being noted 3 weeks after the final immunisation. Data obtained at the same time-point showed decreased IFN-γ and IL-17 responses indicative of a poor Th1 mediated immune response in mice immunised with DODA:TDB liposomes. The multifunctionality of DDA:TDB verses DODA:TDB liposomes was also investigated and showed a significantly stronger ability of DDA:TDB liposomes to induce both multifunctional and central memory T cells. Results not shown here but also undertaken at SSI described equal abilities of DDA:TDB and DODA:TDB liposomes to present antigen to T cells, as measured by their proliferative ability. Therefore whilst membrane rigidity plays a key role in the formation of memory responses, this is not due to differences between rigid and fluid liposomes to present antigen to APCs.

Further to the work described here, recent findings analysing the role of membrane fluidity by comparing the aforementioned fluid DODA:TDB liposomes with rigid DDA:TDB liposomes highlighted the strong immunostimulatory abilities of DDA:TDB liposomes (Christensen et al., 2010b). Whilst the liposomes used in these studies were not produced at Aston University, this work formed part of a collaboration between Aston University, SSI and Arun Kamath and Claire-Anne Siegrist of the University of Geneva. In contrast to using radiolabelling, fluorescence labelling of liposomes and antigen was undertaken and the proportion of each component measured in the dendritic cell (DC) population found in the local draining lymph node. No significant differences in the proportion of local draining lymph node DCs containing DDA:TDB or DODA:TDB liposomes was observed. This is in contrast to our radiolabelled liposome results which show significant variance at all time-points investigated. Examination of the mean fluorescence intensity (MFI) per DC showed that DC uptake of DDA:TDB liposomes was 4-5 fold more efficient than uptake of the fluid DODA:TDB liposomes, indicating that whilst similar numbers of DCs were involved in DDA:TDB or DODA:TDB liposome uptake, DCs that did take up DDA:TDB liposomes took up more per cell. However, liposome uptake is irrelevant if co-administered Ag85B-ESAT-6 antigen is not uptaken by DCs for presentation on MHC molecules. Administration of Ag85B-ESAT-6 with DODA:TDB liposomes led to a significantly higher proportion of DCs containing Ag85B-ESAT-6 in the local draining lymph node 24 hrs p.i. Unfortunately this did not translate to an overall increased percentage of Ag85B-ESAT-6 dose in the lymph node at the same time-point when measured using radiolabelling. The observed discrepancies between radiolabelled and fluorescently labelled vaccine biodistribution in the lymph nodes may simply be due to various cells participating in vaccine uptake; as solely DCs

were analysed in the work conducted by Arun Kamath, passive draining or cellular uptake by cells other than DCs would not be included in the analysis.

In support of the more immunogenic nature of rigid DDA:TDB liposomes compared to fluid DODA:TDB liposomes (as shown by cytokine production and multiparameter flow cytometry), work conducted by Arun Kamath provided evidence of the highly immunostimulatory nature of DDA:TDB liposomes compared to DODA:TDB liposomes. Increased levels of CD40 and CD86 activation markers on dendritic cells were noted, as was CD86 expressed on macrophages. Whilst DODA:TDB liposomes could also increase CD40 and CD86 upregulation compared to naïve controls, DDA:TDB liposomes were superior. As activation of DCs has been shown to increase their turnover rate (Hou and Van Parijs, 2004; Kamath et al., 2002; Obst et al., 2007), these results suggest that DDA:TDB liposomes are superior activators of DCs, very likely due to the lengthened exposure of DCs to Ag85B-ESAT-6 when delivered with DDA:TDB liposomes as opposed to DODA:TDB liposomes.

Chapter 6: Investigating the Role of Liposome Size on the Immunological Properties of an Established Tuberculosis Vaccine Adjuvant

Papers relating to this chapter

Henriksen-Lacey M, Devitt A and Perrie Y. The effect of vesicle size on the biodistribution and immunogenicity of DDA:TDB liposomes. Molecular Pharmaceutics, 2010, *In preparation.*

6.1 Aims

This final chapter documenting experimental findings relates to the effect of liposome size on the resulting biodistribution profile of liposomes and simultaneously injected antigen. The ability of various sizes of liposomes composed of the immunostimulatory combination 'DDA:TDB' were investigated as vaccines for the induction of both humoral and cell-mediated immune responses after immunisation with the tuberculosis antigen Ag85B-ESAT-6.

6.2 Introduction

For the establishment of immunity against a particular pathogen, its antigenic components must enter the antigen presenting cell (APC) so that the antigen can be loaded onto MHC molecules for subsequent presentation to T cells. The same is true for vaccines carrying antigenic components and therefore vaccine design must consider the various methods by which the antigen may enter the cell. As described in Chapter 1, there are numerous uptake mechanisms designed to uptake pathogens and host-derived substances in the nano- (endocytosis) or micro- (phagocytosis) range. Many experiments use fluorescently labelled polystyrene microspheres to investigate particle uptake as they can be purchased in a variety of narrowly defined sizes, after which surface modifications such as addition of peptide antigens, toxoids or immunomodulatory substances can be achieved using conjugation technology. Another commonly used method is the use of size reduction techniques such as sonication, filtration or extrusion, all of which are suitable techniques for liposomes. Whilst the use of filtration and extrusion results in vesicles of a defined size, dependent on the duration and power of sonication or the extrusion filter pore size, the resulting population is not necessarily homogeneous. Furthermore, in the case of extrusion loss of material is also common due to either non-specific or specific interactions with the membrane. Although sonication generally does not lead to any loss of material, there is no physical size limiting device therefore the population of resulting vesicles may be more heterogeneous than observed after extrusion. In addition, the forces involved in sonication may have a more degradative effect on the material.

The role of vesicle size for antigen uptake and activation of the immune response remains debated with many studies offering contradictory indications as to whether smaller or larger vesicles are superior. In fact, it appears that vesicle size plays a significant role in

biasing the immune response towards Th1 or Th2 immunity (Brewer et al., 1998; Fifis et al., 2004; Mottram et al., 2006). Recently Mann and colleagues revisited the Th1/Th2 paradigm using heterogeneous sized bilosomes (liposomes incorporating bile salts) for oral delivery in an influenza model (Mann et al., 2009). In correlation with results from Brewer and colleagues (Brewer et al., 1998), smaller vesicles enhanced Th2 responses whilst larger liposomes induced high levels of IFN-γ and IgG2 antibodies, characteristic of Th1 responses. Importantly, larger liposomes also gave better protection in the ferret challenge model of influenza (Mann et al., 2009). Particle size has also been shown to have a significant effect on vesicle trafficking to lymph nodes and uptake by APCs (Brewer et al., 2004; Foged et al., 2005; Oussoren et al., 1997). Manolova et al. recently described the trafficking of small nanoparticles and virosomes (20-200 nm) to the local draining lymph nodes (LN) after injection into the footpads of mice (Manolova et al., 2008). Although larger (0.5 - 2 μm) particles remained at the site of injection (SOI), only these larger particles were shown to be uptaken by dendritic cells (DCs) at the SOI whereas small (< 200 nm) particles freely drained to the LN wherein they were efficiently uptaken by phagocytic cells (DCs and macrophages). Previous *in vitro* studies investigating the phagocytic abilities of macrophages compared to monocyte-derived DCs have shown that large (4.5 μm) positively charged vesicles are more efficiently phagocytosed than smaller (1 μm) negatively charge vesicles (Thiele et al., 2001). Furthermore, these cationic vesicles are able to induce DC maturation as measured by CD83 expression. As monocyte-derived DCs are noted for their plasticity and ability to accumulate in LNs after infection (Steinman, 2008), vaccines composed of larger cationic liposomes may provide an ideal method to target and stimulate this population of DCs.

The discrepancies noted in comparing studies on particulate size and resulting immune responses are partly due to the variable vesicles compared; whilst a defined size range may be documented, differences in vesicle composition, antigens, animal models (or indeed *in vitro* cell lines) will all have an effect. Therefore, by keeping all of these parameters the same but altering just the vesicle size, it is possible to offer a direct comparison of the effect of vesicle size. It has continually been shown that liposomes composed of DDA and TDB produce a strong antigen depot-effect which is independent on the route of injection (Chapter 3), dependent on the cationic surface charge and antigen adsorption (Chapter 4) and dependent on the rigidity of the liposome bilayer (Chapter 5).

Therefore the aim of this work was to investigate whether rigid cationic liposomes (i.e. DDA:TDB) which were of various size distributions, would have similar abilities to cause an antigen depot-effect at the SOI. To investigate this, DDA:TDB liposomes were produced to three different sizes, termed small, medium and large herein. Small liposomes were produced using the usual lipid-film hydration method followed by a sonication step to reduce the mean vesicle diameter. Medium liposomes were the same as the DDA:TDB liposomes documented in previous chapters. Large liposomes were produced using a novel method effectively leading to increased lipid packing in the bilayers and a larger vesicle size (see Chapter 2, Section 2.2.1 for method). After investigations relating to buffer molarity (Chapter 3) it was discovered that by using a PBS buffer as opposed to Tris buffer the size of the liposomes could be controlled and upon hydration of DDA:TDB lipid films with PBS buffer at 10 mM, large vesicles formed (Chapter 3). Therefore as a final formulation in some of the described studies, extra large DDA:TDB liposomes produced using PBS buffer to hydrate lipid films were also included.

6.3 Results and Discussion

6.3.1 Characterisation of DDA:TDB liposomes of varying size distributions

DDA:TDB liposomes, produced using the lipid-hydration method, were made to four different size ranges by use of sonication (small liposomes), increased lipid packing (large liposomes) or substituting Tris buffer with PBS buffer (10 mM) for rehydration of lipid films (extra large liposomes); medium liposomes were produced without any alteration to the usual technique. Medium DDA:TDB liposomes were slightly larger than that noted in previous chapters (possibly due to use of a different lipid batch) and expressed a mean vesicle size of 685 nm (Figure 6.1, A). Sonication effectively reduced the size range of these medium vesicles to ~ 175 nm, whilst the use of the lipid-packing technique resulted in vesicles approximately 2-fold larger than normal with a mean vesicle size of 1.5 µm (Figure 6.1, A). The largest vesicles produced were on the limit of accurate measurement using the dynamic light scattering technique (DLS), however the value obtained showed vesicles of between 2 – 3 µm formed. The sizes measured using DLS were supported by TEM imaging which also showed the heterogeneous nature of the vesicles produced (Figure 6.2)

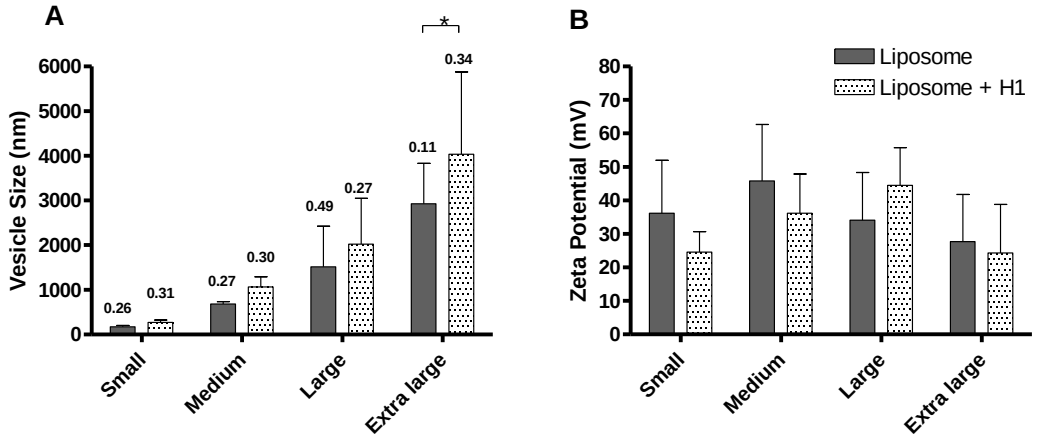

Figure 6.1 Physicochemical characteristics of DDA:TDB liposomes prior and post addition of Ag85B-ESAT-6 antigen. Liposomes were made to four different sizes, termed small, medium, large and extra large followed by addition of Ag85B-ESAT-6 to a final protein concentration of 10 µg/ml liposomes. Vesicle size (A; bars), polydispersity (A; values) and zeta potential (B) were all measured using a Brookhaven ZetaPlus. Results denote mean ± SD of 3 samples. (*p<0.05).

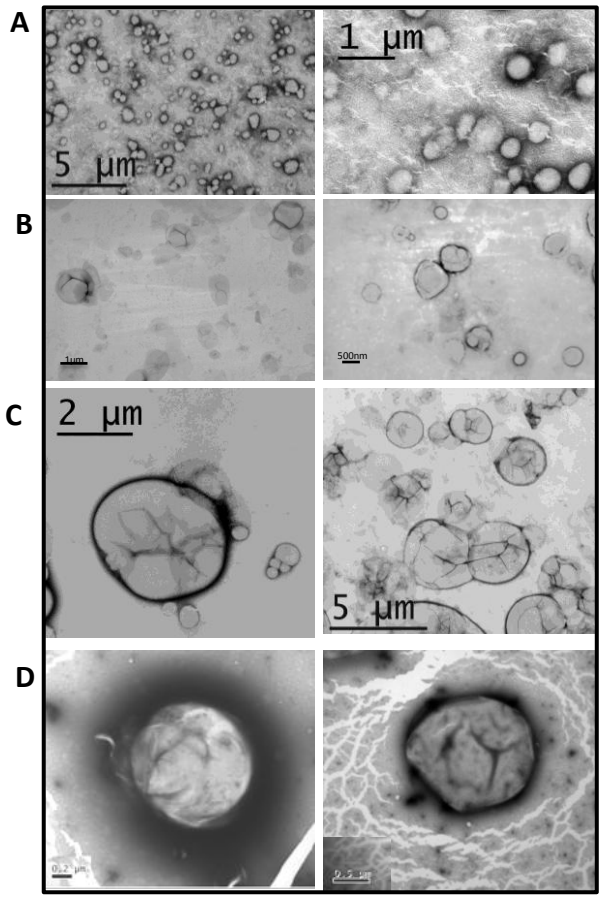

Figure 6.2 Transmission electron microscopy showing liposomes produced to the small (A), medium (B), large (C) and extra large (D) defined size ranges.

It is particularly interesting to note that the method used to produce large liposomes does actually result in vesicles of an increased diameter and not simply vesicle aggregates, which is a difference that DLS cannot differentiate between. All liposomes expressed a positive surface charge which varied between 28 – 47 mV depending on the liposome size; although not significantly different from one another, the most cationic liposomes were of a medium size (+47 mV) whilst extra large liposomes were least cationic (+28 mV) (Figure 6.1).

The stability of these liposomes over a 28-day period when stored at 4 °C and 25 °C was addressed. However, as the extra large liposomes were too large to be accurately measured by DLS, they were not included in this study. Furthermore, medium liposomes have been characterised in Chapters 3 and 5 and have been shown to remain stable over a 56-day period regardless of the storage temperature or presence of Ag85B-ESAT-6. They were therefore not repeated. Figures 6.3 and 6.4 address the changes in physicochemical characteristics noted for small and large liposomes respectively. Whilst small liposomes without Ag85B-ESAT-6 are relatively stable over the 28-day period (Figure 6.3, A,B), addition of Ag85B-ESAT-6 caused liposomes stored at 25 °C to significantly increase in vesicle size becoming approximately 4-fold larger by day 28 post formation. No similar temperature dependent changes were noted for their zeta potential (Figure 6.3, *b*). Large DDA:TDB liposomes also increased in size when stored at 25 °C but not at 4 °C, although it should be noted that it was difficult to measure these vesicle sizes accurately due to the large size of the formulation (Figure 6.4, A, *a*). In general it appears that storage of small and large DDA:TDB liposomes at 4 °C hinders any Ag85B-ESAT-6 antigen-induced variations in vesicle size noted. At higher temperatures (25 °C) thermal instabilities in the liposome bilayer can occur leading to loss of adsorbed Ag85B-ESAT-6 and/or electrostatic-mediated aggregation and potentially fusion of adjacent liposomes. The finding that significant temperature and antigen mediated changes in vesicle size are noted for small and large DDA:TDB liposomes, but not for normal sized DDA:TDB liposomes (Chapter 5, Figure 5.4), also suggests that these small and large DDA:TDB liposomes are more thermodynamically unstable due to their unnatural structure.

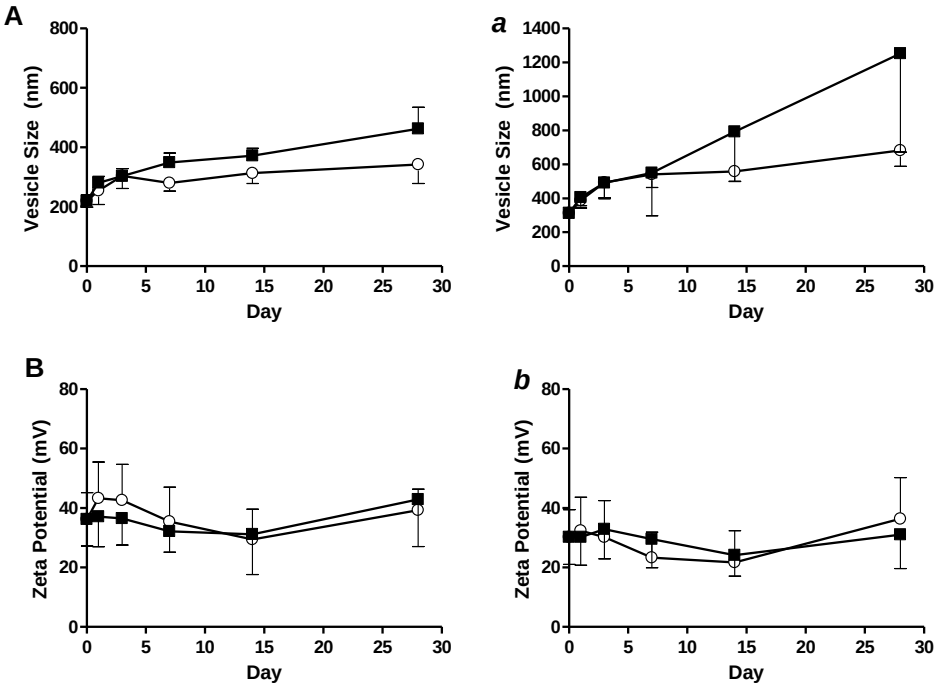

Figure 6.3 Small DDA:TDB liposomes were stored at 4 °C (○) or 25 °C (■) for 28 days. Periodically the vesicle size (A, *a*) and zeta potential (B, *b*) of liposomes both without (A, B) or with Ag85B-ESAT-6 (*a, b*) was measured using a Brookhaven ZetaPlus. Results denote mean ± SD of triplicate samples.

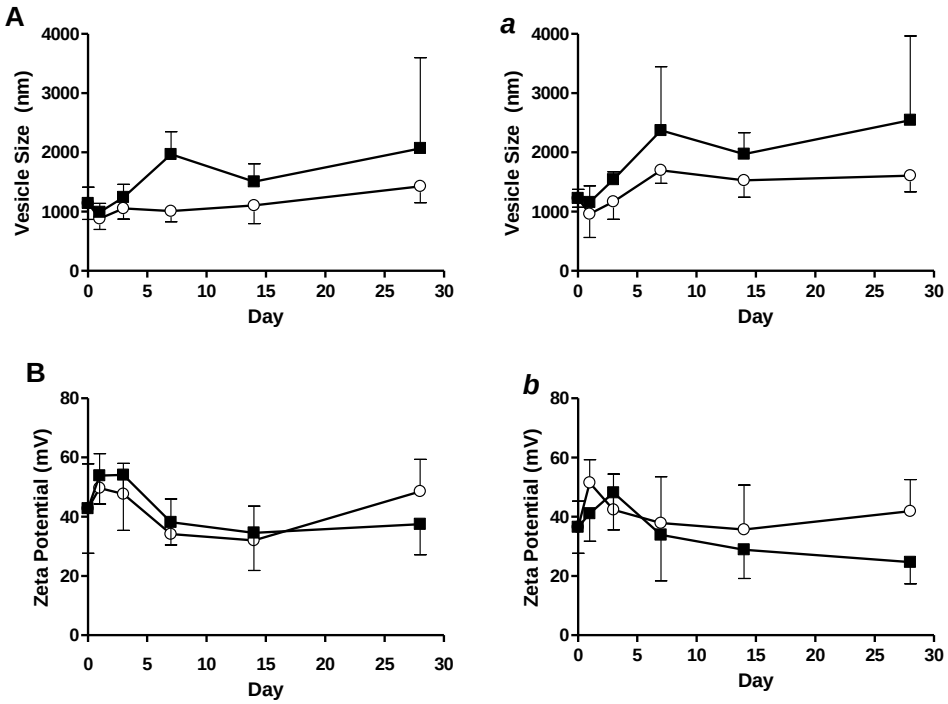

Figure 6.4 Large DDA:TDB liposomes were stored at 4 °C (○) or 25 °C (■) for 28 days. Periodically the vesicle size (A, *a*) and zeta potential (B, *b*) of liposomes both without (A, B) or with Ag85B-ESAT-6 (*a, b*) was measured using a Brookhaven ZetaPlus. Results denote mean ± SD of triplicate samples.

6.3.2 Investigation into the protein adsorption abilities of small and large liposomes

The ability of the small and large DDA:TDB liposomes described above to adsorb antigen were investigated with both radiolabelling and fluorescence labelling techniques. Radiolabelled Ag85B-ESAT-6 was used to determine the proportion of Ag85B-ESAT-6 adsorption and subsequent release in simulated *in vivo* conditions, thereby providing complementary data for biodistribution studies. In addition, fluorescent labelling of liposomal bilayers was used to gain a more visual idea of how protein interacts with the liposomes as well as to support visual data obtained from TEM.

In correlation with the high levels of Ag85B-ESAT-6 adsorption noted for medium DDA:TDB liposomes (Chapters 3, 4 and 5), both small and large DDA:TDB liposomes are capable of adsorbing large amounts of Ag85B-ESAT-6, thereby demonstrating the importance of cationic surface charge and not liposome geometry (Figure 6.5). However it was noted that unlike the medium DDA:TDB liposomes, both small and large liposomes exhibited significantly faster rates of Ag85B-ESAT-6 dissociation upon exposure to 50 % FCS at 37 °C, thereby simulating the *in vivo* environment (Figure 6.5). After 96 hrs in these conditions, ~ 75 % of Ag85B-ESAT-6 remained adsorbed to the liposomes.

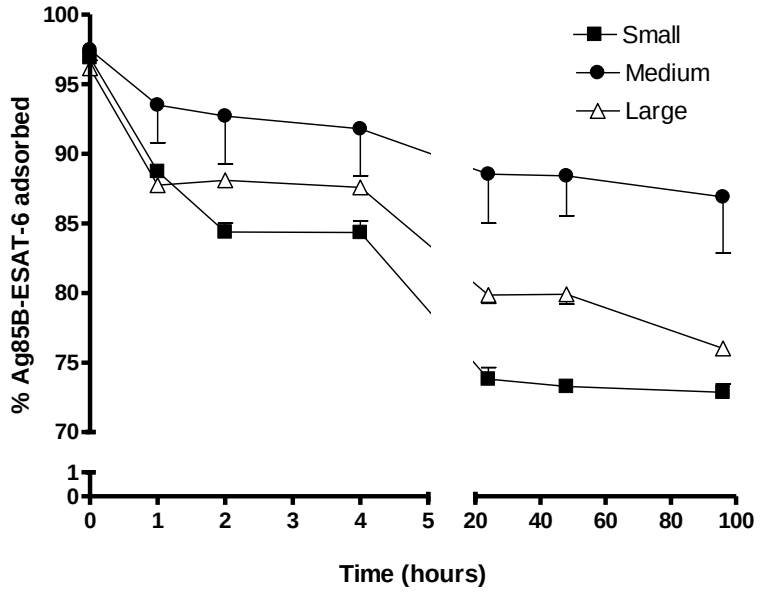

Figure 6.5 Ag85B-ESAT-6 adsorption and release kinetics from small and large DDA:TDB liposomes placed in conditions simulating the *in vivo* environment (37 °C, 50 % FCS). Results denote mean ± SD of triplicate experiments. Medium size liposome data is taken from Figure 3.10, Chapter 3.

Rather than the physical geometry of the liposome, these increased rates of antigen loss may have been due to batch variation in the radiolabelled Ag85B-ESAT-6 as both small and large liposomes follow a very similar kinetic pattern. It is also likely that the liposomes exhibited a type of 'burst-release' pattern as after 24 hrs in simulated *in vivo* conditions the antigen release stabilised.

In order to gain a clearer understanding of the geometry of the large DDA:TDB liposomes as well as to further investigate how protein affects these structures, both small and large DDA:TDB liposomes were fluorescently labelled using Rho-DPPE which incorporates into the lipid bilayer. Liposomes were then mixed with FITC-labelled ovalbumin and viewed under the Rho- and FITC- filters of a fluorescent microscope. In general, small liposomes were too small to be clearly defined, even when using the highest magnification (x100). Figure 6.6 shows a series of z-stack images of small and large DDA:TDB liposomes (Rho-labelled, red) with added OVA (FITC-labelled, green) to a final concentration of ~ 100 µg/ml. By using z-stacks it is possible to see the multilamellar nature of the large liposomes (columns B and C) whereas small liposomes appear to be unilamellar (column A). Furthermore, the z-stack images give a good cross-sectional view of how protein associates with cationic liposomes; it appears that protein is also within the liposomes although this could be due to invaginations in the liposome bilayer and consequently depending on the level of the z-stack it may appear that protein is within the vesicles when it is actually still surface bound.

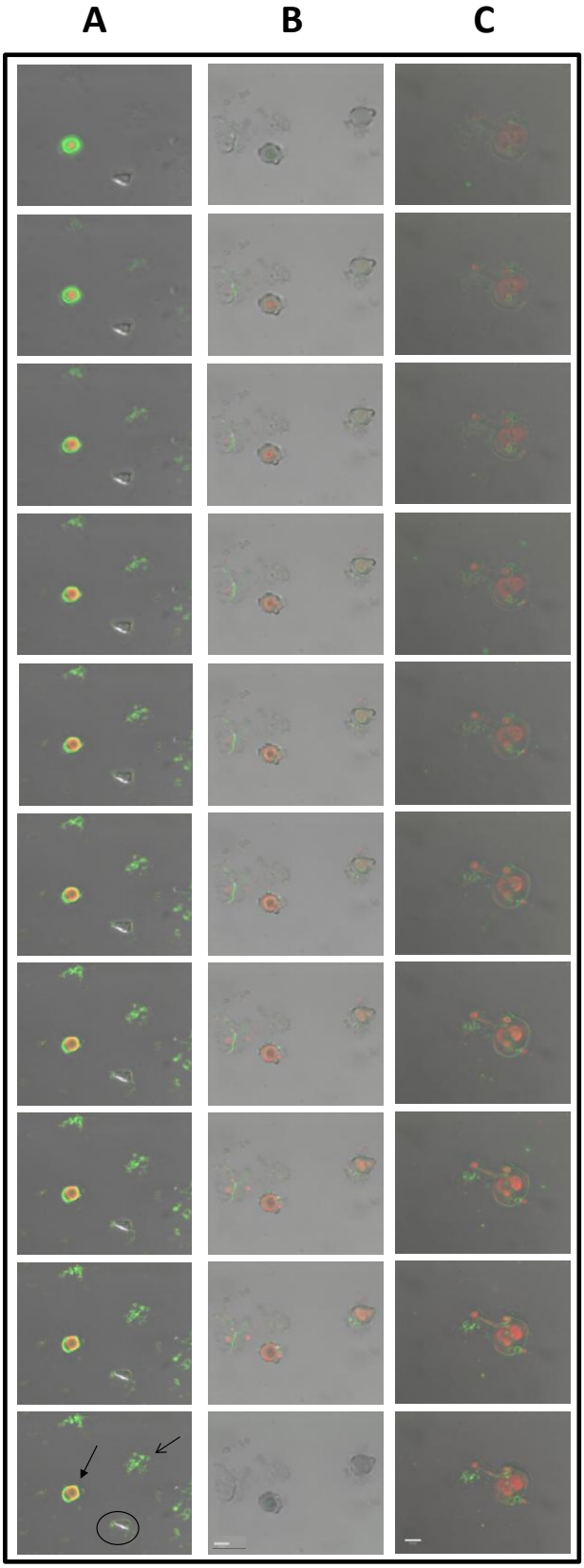

Figure 6.6 A series of z-stack images showing small (column A) and large (columns B and C) Rho-labelled DDA:TDB liposomes with FITC-labelled OVA. The bottom left image marks the presence of liposomes (closed arrow), aggregates of protein ± liposomes (open arrow) and an artefact (circle).

6.3.3 Attempted determination of liposome-mediated intracellular lysosomal activity

The NAG assay can be used to quantify cellular lysosomal activity due to liposomal uptake. As lysosomes are involved in the breakdown of extracellular matter taken up via an endocytotic route, quantification of the amount of NAG present intracellularly may give an indication to the endocytotic ability of the cell. NAG is one of the enzymes involved in the degradation of glycolipids and proteins and therefore is of interest in studies in which liposomes adsorbing protein are added to cells. There is little literature on the use of the NAG assay to measure cellular activity and most research is based on its use to measure elevated levels of NAG present in urine of people with renal disorders.

Attempts to measure NAG in the supernatant of cells exposed to various concentrations of liposomes were unsuccessful (Figure 6.7, A). Firstly, the absorbance values derived from the assay were very low (< 0.2 units) suggestive of background levels. Control wells set up as positive (cells lysed with Triton-X100) and negative (no liposomes) also gave very low absorbance readings ranging between 0.143 and 0.192 (results not shown). Simultaneously conducted experiments to determine cell viability upon addition of small or large DDA:TDB liposomes at 4 °C or 37 °C showed that control wells in which nothing had been applied were capable of NR uptake whilst those which had been lysed by Triton-X100 released all their NR into the surrounding medium (Figure 6.7, B). This confirms the ability of Triton-X100 to successfully lyse cells as well as confirming the ability of cells to pinocytose. The results relating to the NAG assay therefore suggest that there was no NAG present in the cells as all the non-cellular controls relating to the assay effectiveness (p-Nitrophenol standard and NAG control enzyme) worked very well with high absorbance values derived (between 0.7 and 2.4 depending on the control). Whilst Figure 6.7, A shows slightly higher absorbance values for cells in which liposomes were applied at 4 °C as opposed to 37 °C, when the controls are considered these variances are insignificant.

To confirm that liposomes were indeed taken up by cells, fluorescently labelled small and large liposomes associated with FITC-labelled OVA antigen were added to cells and images taken. As seen in Figure 6.8, red (indicative of liposomes) and green (indicative of OVA) staining can clearly be seen within the cells and both are uptaken simultaneously by the cells (Figure 6.8, C).

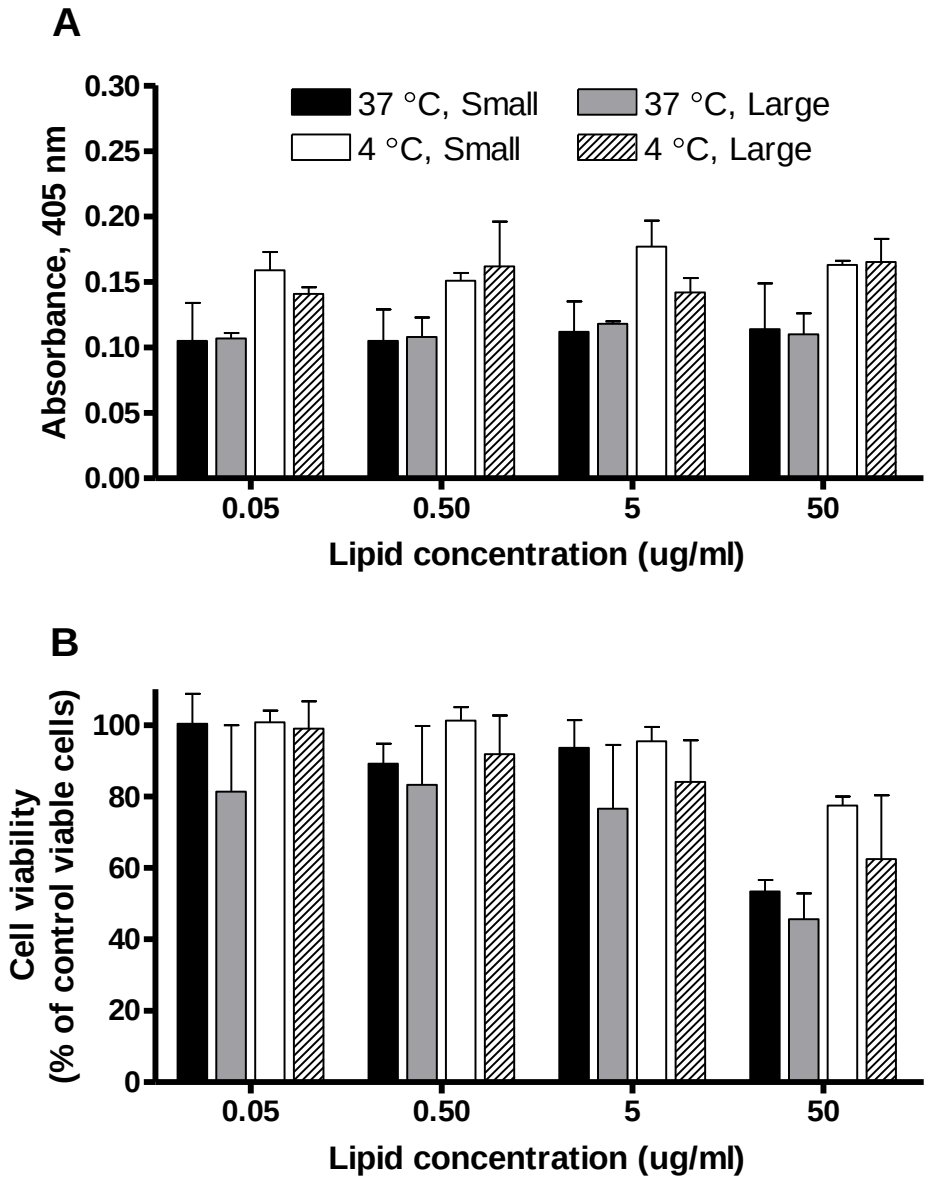

Figure 6.7 Small and large DDA:TDB liposomes were added to J774 cells at concentrations ranging from 0.05 – 50 µg/ml and left to incubate with the cells at 4 °C or 37 °C for 4 hrs. A; supernatants were removed and cell monolayers lysed. The supernatant was subsequently assayed for the presence of NAG by measurement of a colour change detected at 405 nm. B; cells were assayed directly for NR uptake (measured at 560 nm) as an indicator of cell viability. Results denote the mean ± SD.

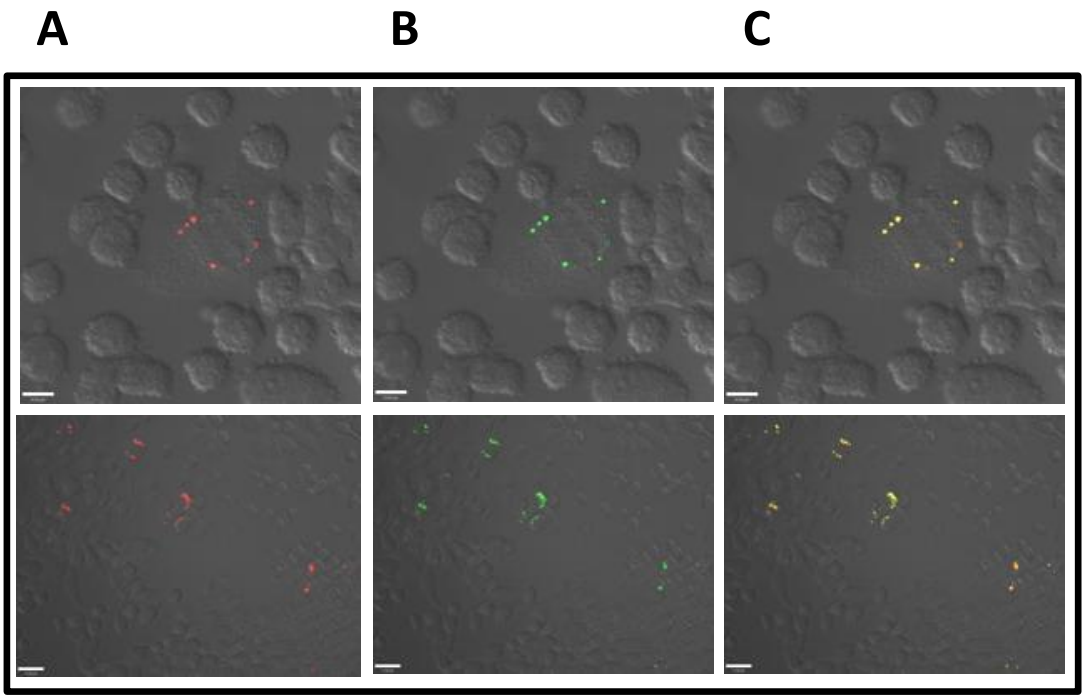

Figure 6.8 DDA:TDB liposome uptake by THP-1 cells. Liposomes were labelled with rhodamine-DPPE (A; red) and mixed with FITC-OVA (B; green); photos of liposomes, protein and cells were taken using a fluorescent microscope and overlaid with phase contrast images to produce the above images (C; liposome+OVA+phase contrast).

6.3.4 Biodistribution of small, medium and large DDA:TDB liposomes

To determine the role of liposome size on the antigen (and liposome) depot-effect at the SOI, DDA:TDB liposomes were produced with the inclusion of ^{3}H-DPPC in the bilayer membrane and ^{125}I-labelled Ag85B-ESAT-6 antigen as previously outlined (Chapter 2, sections 2.2.2 and 2.4.3). The SOI and various other tissues were collected and the percentage of the administered dose (% dose) quantified. Figure 6.9 shows the proportion of liposomes (A) and Ag85B-ESAT-6 antigen (B) retained at the SOI following i.m injection. Regardless of the liposome size (small, medium or large), all liposomes were well retained with between 40 – 50 % of the injected dose recovered after 14 days. It is interesting to note that even at the earliest time-point of 6 hrs (0.25 days) the entire dose is not recovered. Although this may be due to passive draining of the liposomes from the injection site, it is more likely to be due to difficulties collecting the entire dose. Analysis of the proportion of Ag85B-ESAT-6 recovered shows very high retention (~ 80 % for small liposomes) at the earliest time-point of 6 hrs, followed by a gradual decrease reaching

levels of between 1 – 10 % of the dose, depending on the liposome size (Figure 6.9, B). Significantly different results are only noted between small and medium liposomes analysed 4 days p.i ($p<0.01$) suggesting that small liposomes are able to cause a longer Ag85B-ESAT-6 retention profile at the SOI. This is also supported by Ag85B-ESAT-6 recovery on day 14 p.i being the highest (10.6 % dose) after injection with small DDA:TDB liposomes (Figure 6.9, B).

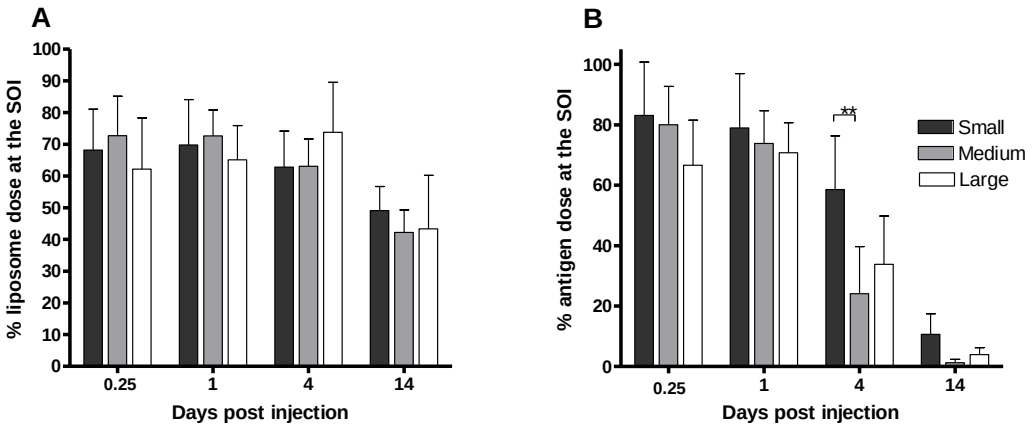

Figure 6.9 Pharmacokinetics of small, medium and large DDA:TDB liposomes administered with Ag85B-ESAT-6 antigen via the i.m route. Tissue from the site of injection (SOI) was collected 6 hrs (0.25 days), 1 day, 4 days or 14 days p.i. Results denote mean ± SD of 3 or 4 mice. **p<0.01.

In addition to investigating the ability of different sized DDA:TDB liposomes to retain Ag85B-ESAT-6 at the SOI, various tissues were assayed and the % dose of both liposome and antigen components determined. The results show that vesicle size plays a role in the clearance of liposomes to the local popliteal lymph node (PLN) with larger liposomes appearing in the PLN faster than small liposomes (Figure 6.10, A). Furthermore, small liposomes are also detected in the spleen at significantly lower levels than their medium or large counterparts (Figure 6.10, B). Interestingly, significantly higher quantities of liposomes are detected in the PLN as opposed to the spleen even though the PLN is considerable smaller (the mean spleen weight is ~ 80 mg whilst a popliteal lymph node weighs ~ 1-2 mg).

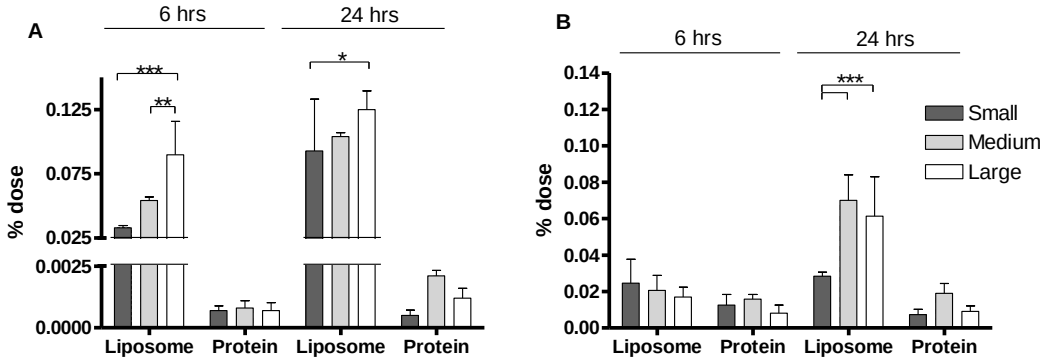

Figure 6.10 Biodistribution of DDA:TDB liposomes (small, medium or large) administered with Ag85B-ESAT-6 antigen (protein). Vaccines were given via the i.m route and tissues including the popliteal lymph node (PLN) (A) and spleen (B) collected 6 or 24 hrs p.i. Results denote mean ± SD of 4 mice. *p<0.05, **p<0.01, ***p<0.001.

6.3.5 Studies addressing immunogenicity of various sizes of DDA:TDB liposomes

The aim of the study was to determine whether different sized DDA:TDB liposomes (small, medium, large and the previously described extra large) would have varying abilities to immunise mice and whether a polarised Th1 or Th2 immune response would ensue, based on previous reports showing liposome size to be a determining factor (Brewer et al., 1998; Mann et al., 2009).

6.3.5.1 Antibody responses against adjuvanted Ag85B-ESAT-6

The ability of small, medium, large and extra large DDA:TDB liposomes to induce IgG (total), IgG1 and IgG2 antibody isotypes was investigated using ELISAs. Blood was collected on days 0, 13, 28, 36 and 49 and mice were injected with vaccines on days 1, 14 and 29. As expected, all PBS injected mice (naïve control group) did not mount any measurable IgG response over the experimental period (Figure 6.11). The results obtained for ICF and liposome immunised mice were not significantly different from each other however there were notable trends (Figure 6.11):

- In general, mice immunised with liposome adjuvanted Ag85B-ESAT-6 mounted total IgG and IgG2 responses to higher titres compared to ICF immunised mice.
- Furthermore, in the case of IgG2, two doses of liposome adjuvanted Ag85B-ESAT-6 vaccine were sufficient to mount the maximum IgG2 titre.

- Finally, for ICF immunised mice, three immunisations were required to reach maximal IgG2 titres (Figure 6.11, C).

Whilst it is difficult to draw any conclusions with regards to the efficiency of the different sized liposomes to induce humoral immune responses, all liposome vaccine formulations induced high levels of both IgG1 and IgG2 antibodies. The results observed do not show any Th1 or Th2 antibody bias as a result of variable liposome size but instead show a mixed Th1/Th2 antibody response unlike the predominantly Th2 response noted upon immunisation with currently used aluminium salt adjuvants.

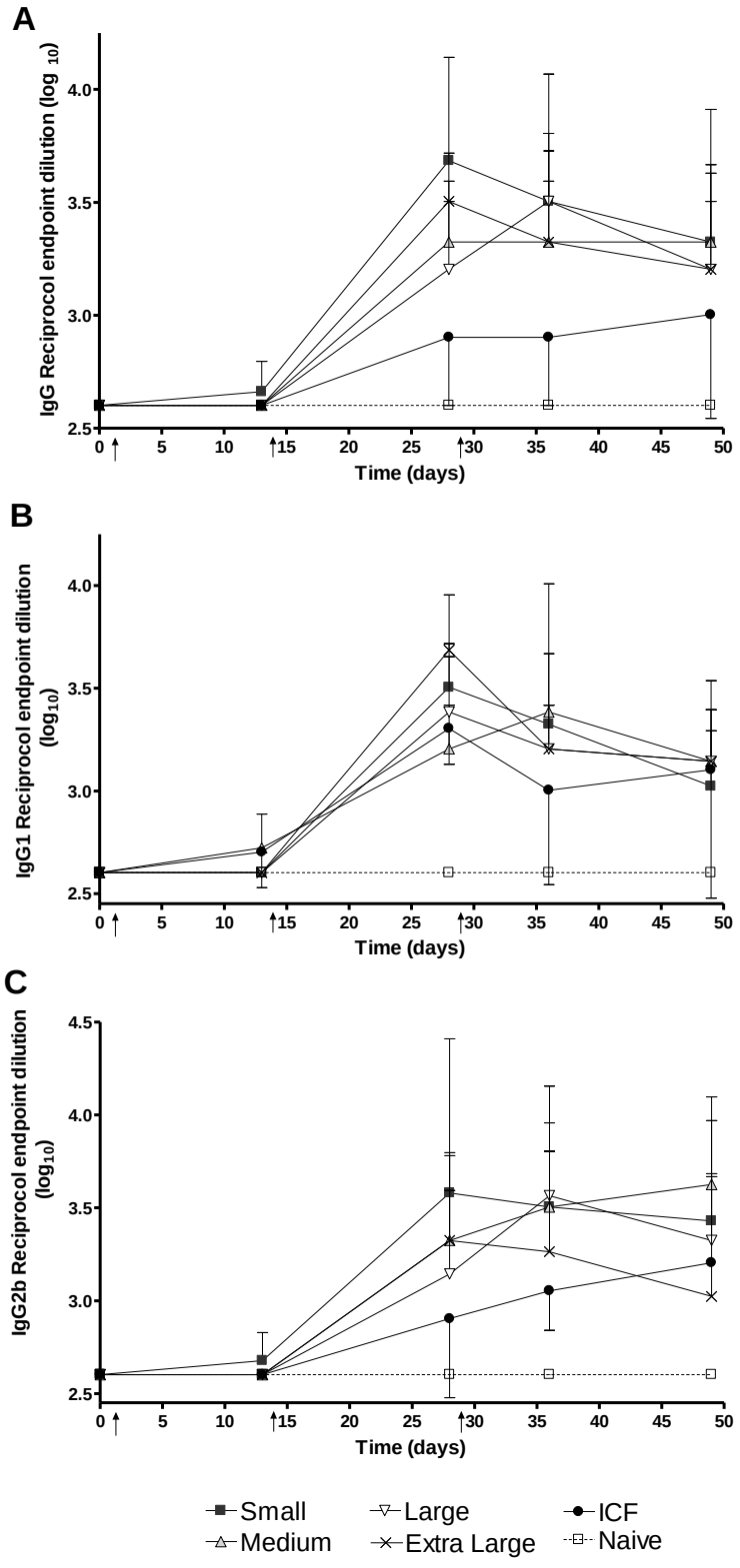

Figure 6.11 Antibody responses detected in the serum of immunised mice injected on days 1, 14 and 29 (depicted with arrows) with various liposomal vaccine formulations adsorbing Ag85B-ESAT-6 antigen. Mice were bled on days 0, 13, 28, 36 and 49 and IgG (A), IgG1 (B) and IgG2b (C) titres measured. Results show the mean ± SD reciprocal endpoint dilution (log_{10}) of 3 (Incomplete Freund's adjuvant, ICF), 4 (naïve unimmunised) or 5 (liposomes) mice.

6.3.5.2 Splenocyte proliferation *ex vivo* in response to Ag85B-ESAT-6

On day 49 all mice were terminated and their spleens removed for assessment of proliferative ability in response to Ag85B-ESAT-6 antigen. Splenocytes were restimulated with 0.05, 0.5 and 5 μg/ml Ag85B-ESAT-6 in addition to the positive control stimuli, ConA (5 μg/ml). Figure 6.12 shows the proliferative ability of splenocytes in response to ConA. All mice responded to ConA although 7 mice showed poor levels of proliferation which resulted in ~ 1000 fold less ^{3}H-thymidine uptake compared to naïve unimmunised mice. The average ^{3}H-thymidine uptake was between 10,000 – 100,000 counts per minute (CPM). Splenocytes derived from unimmunised mice or those immunised with extra large liposomes or ICF responded to nearly equal proliferative levels with a very small standard deviation noted between mice (Figure 6.12).

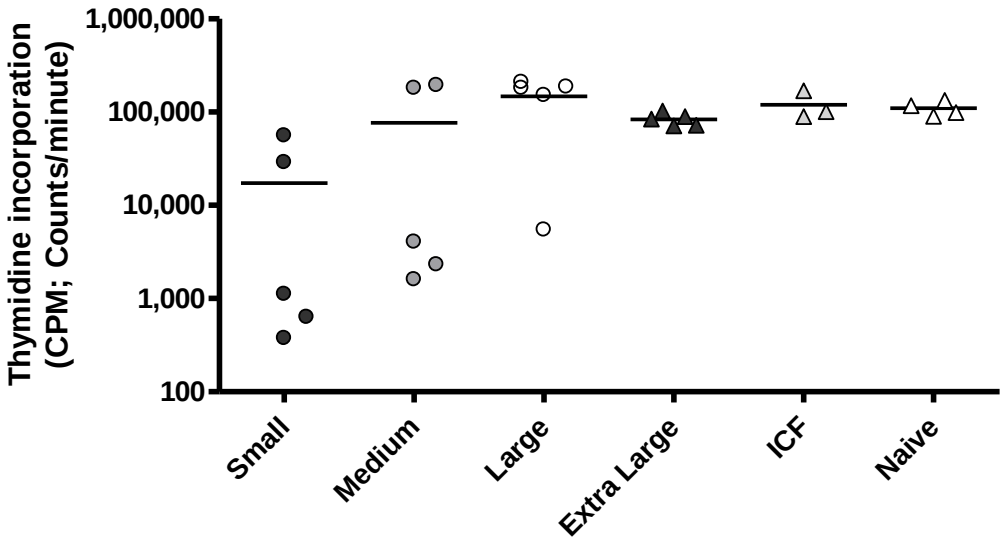

Figure 6.12 Splenocyte proliferation in response to the positive control stimuli ConA. Splenocytes were derived on day 49 of the study and restimulated *ex vivo* with ConA (5 μg/ml) to induce proliferation. ^{3}H-thymidine was applied to the cells 72 hrs after exposure to ConA. After a further 24 hrs cells were harvested and the proportion of ^{3}H-thymidine incorporated in the cells measured using standard scintillation counting. Results are shown for individual mice as the mean counts per minute (CPM) of triplicate wells of a 96-well plate.

In addition to inducing splenocyte proliferation with the non-specific stimulator ConA, splenocytes were also exposed to previously encountered Ag85B-ESAT-6 antigen. Figure 6.13 shows the results for individual spleens whilst Figure 6.14 gives an overview for all liposomal vaccine formulations. The results expressed in Figure 6.13 outline two

immediate findings; firstly baseline levels of splenocyte ^{3}H-thymidine uptake are < 2000 CPM as proliferation (or rather uptake) is still noted in the absence of Ag85B-ESAT-6 restimulation (Figure 6.13, A); secondly, all unimmunised and ICF immunised mice did not respond to Ag85B-ESAT-6 restimulation to levels above background (Figure 6.13). With regards to spleens derived from mice immunised with liposomal vaccines (small, medium, large or extra large DDA:TDB liposomes adsorbing Ag85B-ESAT-6 antigen), in general there was a liposome size and Ag85B-ESAT-6 concentration dependent increase in proliferation noted (Figure 6.14). Whilst small DDA:TDB liposomes failed to induce levels of proliferation deemed significantly higher than naïve unimmunised mice upon restimulation with 0.05 or 0.5 μg/ml Ag85B-ESAT-6 (Figure 6.13, B and C), all other formulations could do so and showed increases in proliferation to be related to increases in liposome size. The same liposome size-dependent increase in splenocyte proliferation was noted upon restimulation with 0.5 and 5 μg/ml Ag85B-ESAT-6 (Figure 6.13, C, D). Immunisation of mice with small DDA:TDB liposomes did induce significant levels of splenocyte proliferation however the highest concentration of Ag85B-ESAT-6 for restimulation was required (5 μg/ml) (Figure 6.13, D). Regardless of the concentration of Ag85B-ESAT-6 used to restimulate splenocytes, no significant differences in proliferation were noted between medium and large DDA:TDB liposomes (Figure 6.13). Furthermore, irrespective of the Ag85B-ESAT-6 restimulation concentration, mice immunised with extra large DDA:TDB liposomes responded with the highest proliferative ability (Figures 6.13, 6.14). Taken together, these results show a liposome size-dependent immunising ability of DDA:TDB liposomes to induce splenocyte proliferation upon restimulation with previously experienced Ag85B-ESAT-6 antigen.

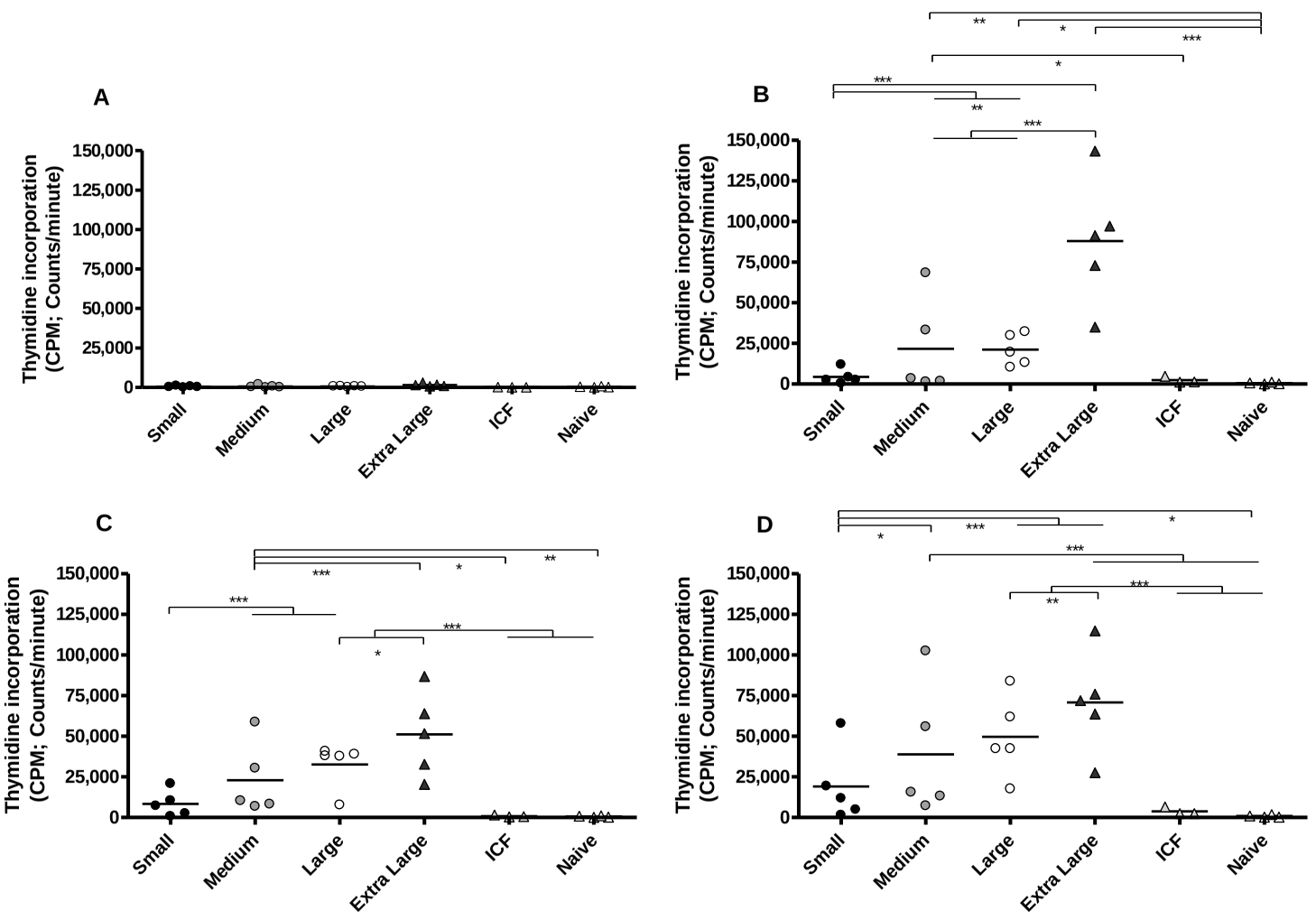

Figure 6.13 Splenocyte proliferation in response to 0, 0.05, 0.5 or 5 µg/ml Ag85B-ESAT-6 (A, B, C or D respectively). Splenocytes were derived on day 48 of the study and restimulated *ex vivo* with Ag85B-ESAT-6 to induce proliferation. ^{3}H-thymidine was applied to the cells 48 hrs after exposure to Ag85B-ESAT-6. After a further 24 hrs cells were harvested and the proportion of ^{3}H-thymidine incorporated in the cells measured using standard scintillation counting. Results are shown for individual mice as the mean counts per minute (CPM) of triplicate wells of a 96-well plate. *p<0.05, **p<0.01, ***p<0.001.

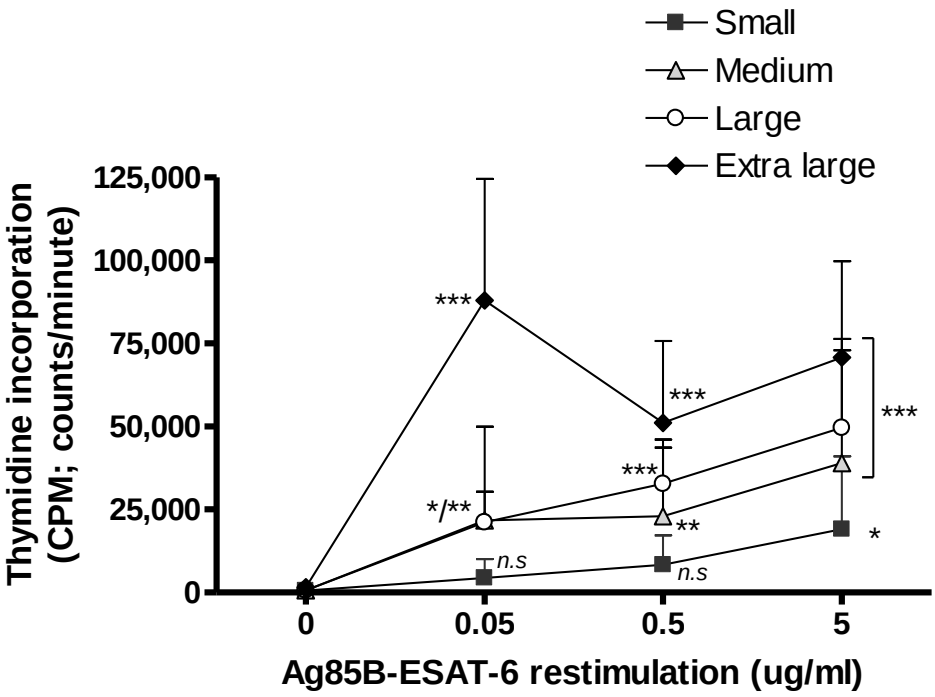

Figure 6.14 Overview of splenocyte proliferation measured using ^{3}H-thymidine incorporation after restimulation *ex vivo* with Ag85B-ESAT-6 at concentrations ranging between 0 – 5 µg/ml. Significance is measured against naïve unimmunised mice (not shown). *p<0.05, **p<0.01, ***p<0.001. The "*/**" noted at 0.05 µg/ml Ag85B-ESAT-6 restimulation refers to large (*) and medium (**) liposomes.

6.3.5.3 Cytokine production from *ex vivo* restimulated splenocytes

The supernatants of restimulated splenocytes were assayed for the presence of cytokines IL-2, IL-5, IL-6, IL-10 and IFN-γ. The same experimental setup as used to determine splenocyte proliferation was used with Ag85B-ESAT-6 being applied to cells at 0.05, 0.5 and 5 µg/ml, in addition to ConA stimulation as a positive control. Whilst Figures 6.15 to 6.19 show cytokine production for individual spleens, Figure 6.20 gives a conclusive overview of the results derived from each experimental group.

Figure 6.15 shows the production of IL-2 from splenocytes in the absence of a stimulant (A), after restimulation with Ag85B-ESAT-6 at 0.05, 0.5 or 5 µg/ml (B, C and D respectively), and finally in the presence of ConA (E). Baseline levels of IL-2 were approximately 230 pg/ml for all immunisation groups investigated (Figure 6.15, A). Upon re-stimulation of splenocytes with Ag85B-ESAT-6 there were significant increases in IL-2 production for all liposome-injected mice, regardless of liposome size; naïve unimmunised mice and ICF

immunised mice did not respond to Ag85B-ESAT-6 at any of the concentrations added. Between the liposome-injected groups the only trend noted was an approximate 2-fold decrease in IL-2 production by splenocytes derived from mice immunised with extra large liposomes. This was significant compared to IL-2 production by medium and large liposome immunisation groups upon restimulation with the highest Ag85B-ESAT-6 concentration (Figure 6.20, A). Small, medium and large liposome injected mice all produced between 1000 – 2600 pg/ml IL-2 when restimulated with 0.05 - 5 µg/ml Ag85B-ESAT-6. The use of ConA as a positive control resulted in significant IL-2 production only from small liposome injected mice (Figure 6.15, E).

IL-2 is commonly described as a Th1 mediating cytokine and has roles as both a pro- and anti-inflammatory cytokine. It is an important mediator of T cell expansion upon TCR and antigen binding and has been shown to act in an autocrine manner stimulating further IL-2 receptor expression, the activation of which leads to induction of effector and regulatory T cells (Lan et al., 2008). With this in mind, the low IL-2 production noted from Ag85B-ESAT-6 restimulated splenocytes derived from ICF immunised mice suggests a poor ability of ICF to present Ag85B-ESAT-6 and prime T cells. Similarly, splenocytes derived from mice immunised with extra large liposomes show the lowest IL-2 production upon Ag85B-ESAT-6 restimulation therefore indicating poorer Ag85B-ESAT-6 presenting abilities of this vaccine.

The next cytokine investigated was IL-5 which plays an important role in steering naïve T cells towards a Th2 phenotype. IL-5 is present in high amounts in pro-inflammatory disorders such as allergies and asthma. IL-5 is also one of the principle cytokines used to detect immune responses after experimental administration of mineral salt adjuvants due to their Th2 polarising abilities. Figure 6.16 shows IL-5 production from splenocytes in the absence of a stimulant (A), after restimulation with Ag85B-ESAT-6 at 0.05, 0.5 or 5 µg/ml (B, C and D respectively), and in the presence of ConA (E). Unlike IL-2 production, there appeared to be no 'background' level of IL-5 production as many splenocyte cultures were totally unresponsive even in the presence of Ag85B-ESAT-6 or ConA stimuli. Whilst levels of IL-5 production were approximately 10-fold lower than IL-2 production in responding splenocyte cultures, a similar pattern was noted with splenocytes derived from extra large liposome injected mice being the poorest cytokine producers when compared to the other liposome injected mice. This was considered significant when compared to the large liposome group at the highest Ag85B-EAT-6 restimulation concentration (Figure 6.20, B).

Levels of IL-5 from Ag85B-ESAT-6 restimulated splenocytes derived from ICF injected or unimmunised mice were non-existent (< 15 pg/ml) even in the presence of ConA stimulation. ConA stimulation induced the highest IL-5 production from splenocytes derived from small liposome injected mice (~ 320 pg/ml) (Figure 6.16, E) although this was deemed non-significantly different from other liposomal formulations. Taken together, these results suggest that when combined with Ag85B-ESAT-6, DDA:TDB liposomes and ICF do not stimulate the Th2 arm of the immune response, however the poor IL-5 response noted upon ConA stimulation may also suggest a bias in this mouse strain towards Th1 responses (Charles et al., 1999).

The following cytokine investigated was IL-6 which is an important cytokine for B cell differentiation towards antibody secreting plasma cells, as well as playing a role in the differentiation of T cells to a Th1 or Th2 phenotype (Jones, 2005). IL-6 is therefore an important cytokine for detection of humoral immune responses as well as cell-mediated immunity. Whilst IL-6 has been implicated in IL-2 promotion and therefore Th1 immune responses, the critical finding that IL-6 promotes autocrine actions of IL-4 (Th2 cytokine) as well as inhibiting IFN-γ production suggests its involvement in predominantly Th2 responses (Diehl and Rincón, 2002; Dienz and Rincon, 2009). However it does appear to be a rather complex relationship, also highlighted by the recent finding that IL-6 is involved in naïve T cell differentiation towards the Th17 phenotype (McGeachy and Cua, 2008).

IL-6 was detected in the supernatant of splenocytes derived from mice immunised with and restimulated with the same experimental conditions as noted for IL-2 and IL-5. Figure 6.17 shows their response in the absence (A) or presence of Ag85B-ESAT-6 (B, C, D) or ConA (E). Unstimulated splenocyte IL-6 production was between 20 – 250 pg/ml with no particular trend noted between immunisation groups (Figure 6.17, A). These background levels of IL-6 were maintained in splenocytes derived from ICF immunised and naïve unimmunised mice upon restimulation with Ag85B-ESAT-6. In contrast, stimulation of the same splenocyte populations with ConA caused ~ 2-fold higher levels of IL-6 production highlighting the ability of ConA to restimulate splenocytes irrespective of the vaccine used to immunise mice with (Figure 6.17, E). With regards to splenocytes derived from liposome-immunised mice, whilst no trend was noted between the liposomal groups, all groups responded to Ag85B-ESAT-6 restimulation producing levels of IL-6 approximately 3 - 6 fold higher than ICF or naïve groups. Peak IL-6 production was noted by splenocytes derived from mice immunised with medium and large liposomes and restimulated with

the highest Ag85B-ESAT-6 concentration (1100 pg/ml; Figure 6.17, D). No significant differences in IL-6 production were observed between liposome formulations (Figure 6.20, C).

The final cytokine of the interleukin family investigated was IL-10 which is also known to have dual Th1/Th2 differentiating abilities as well as being involved in establishing humoral immune responses (Mocellin et al., 2004). To further complicate matters, IL-10 production has been detected from IL-6 stimulated Th17 cells and has been shown to regulate Th1 and Th17 responses in a type of negative feedback loop thereby preventing host-derived excessive inflammatory responses (Heo et al., 2010; McGeachy and Cua, 2008). In addition to this important link between IL-6 and IL-10 responses, IL-10 has also been shown to be inhibitory to IL-2 and IFN-γ production from Th1 cells (Fiorentino et al., 1989), as well as inhibiting cell proliferation (Mocellin et al., 2004). Analysis of IL-10 production from splenocytes is therefore of particular interest with regards to their proliferative ability (discussed in section 6.2.5.2).

Figure 6.18 shows IL-10 production from splenocytes derived from naïve unimmunised mice, ICF and liposomal immunised mice, either in the absence (A) or presence of Ag85B-ESAT-6 (B, C, D) or ConA (E). Generally low but variable of IL-10 were detected from unstimulated splenocytes with no trend or significant differences between immunisation groups noted (Figure 6.18, A). In contrast, the use of ConA to stimulate splenocytes to produce cytokines was successful as high levels of IL-10 were noted from splenocytes derived from liposome immunised mice (~ 1100 pg/ml; Figure 6.18, B). Levels of IL-10 from ConA stimulated ICF and naïve splenocytes were lower (~ 200 pg/ml) but were still significantly higher than background (i.e no stimulation). Re-exposure of liposome immunised mouse splenocytes to Ag85B-ESAT-6 lead to the interesting finding that IL-10 production was liposome size dependent; regardless of the Ag85B-ESAT-6 restimulation concentration, a trend was noted whereby splenocytes derived from mice injected with the smallest liposomes produced the highest levels of IL-10 (~ 350 pg/ml) whereas those receiving the largest of liposomes produced the least IL-10 (~ 80 pg/ml). Whilst these results were only deemed significant between small versus large and extra large groups when restimulated with 0.05 µg/ml Ag85B-ESAT-6 (Figure 6.20, D), the trend was clearly visible and applied over the three concentrations of Ag85B-ESAT-6 used to restimulated splenocytes (Figure 6.18, B, C, D). Whilst there was no similar liposome size-dependent trend noted in the production of IL-2, IL-5 or IL-6, liposome size (and Ag85B-ESAT-6

restimulation concentration) had a significant effect on the proliferative ability of splenocytes (Figure 6.14). Interestingly, the relationship between IL-10 production and splenocyte proliferation as a result of immunisation with various sized liposomes was an inverse one: high IL-10 production gave low proliferation and vice versa. This result correlates nicely with described cell proliferation inhibitory actions of IL-10 (Mocellin et al., 2004).

The last and perhaps the most important cytokine investigated was IFN-γ which is a commonly used marker to determine the efficacy of tuberculosis vaccines (Agger and Andersen, 2001). In the same format as for the previous cytokines, Figure 6.19 shows IFN-γ production from unstimulated (A), and (re-)stimulated (B, C, D, E) splenocytes derived from naïve, ICF or liposomal immunised mice. Interestingly no splenocyte samples produced IFN-γ when cultured in the absence of Ag85B-ESAT-6 or ConA (Figure 6.19, A). This was the only cytokine studied for which this was noted and consequently suggests its production is infection-mediated and highly dependent on splenocyte activation. In accordance with this thought, splenocytes derived from naïve unimmunised mice also failed to produce IFN-γ when exposed to Ag85B-ESAT-6. Unexpectedly splenocytes derived from ICF immunised mice which had been previously exposed to Ag85B-ESAT-6 *in vivo* also failed to recognise Ag85B-ESAT-6 upon re-exposure and no IFN-γ response was detected across the range of concentrations tested. Both ICF immunised and naïve unimmunised mouse splenocytes did however respond to the positive stimuli ConA with IFN-γ produced to ~1500 pg/ml (Figure 6.19, E). Without exception, production of IFN-γ from splenocytes derived from liposomal immunised mice was significantly higher than any other cytokine investigated; on average between 7000 - 27000 pg/ml IFN-γ was detected after re-exposure to Ag85B-ESAT-6. Splenocytes derived from mice immunised with medium sized liposomes resulted in the highest mean IFN-γ production regardless of the concentration of Ag85B-ESAT-6 used for restimulation (Figure 6.19, B, C D). The same was also true upon exposure to ConA as opposed to Ag85B-ESAT-6 (Figure 6.19, E). Medium sized liposomes induced significantly higher ($p<0.01$ and $p<0.001$) levels of IFN-γ from restimulated splenocytes (0.5 µg/ml Ag85B-ESAT-6) compared to splenocytes derived from small, medium and large liposomes (Figure 6.20, E).

IFN-γ is an important correlate of protective immunity and numerous TB vaccine studies have shown IFN-γ production and vaccine efficacy to be analogous (Agger and Andersen, 2001). In terms of TB infection, IFN-γ plays an important role in both primary and

subsequent *Mycobacterium tuberculosis* (*M.tb*) infection. Mice lacking IFN-γ producing cells are susceptible to primary *M.tb* challenge whilst upon secondary infection IFN-γ is capable of limiting *M.tb* growth (Goldsack and Kirman, 2007; Serbina and Flynn, 2001). Importantly however, not all vaccines which offer protection against *M.tb* challenge also induce high levels of IFN-γ (Badell et al., 2009) therefore highlighting the need to look at the overall immune response including other cytokines, antibody isotypes and ultimately protection in challenge studies.

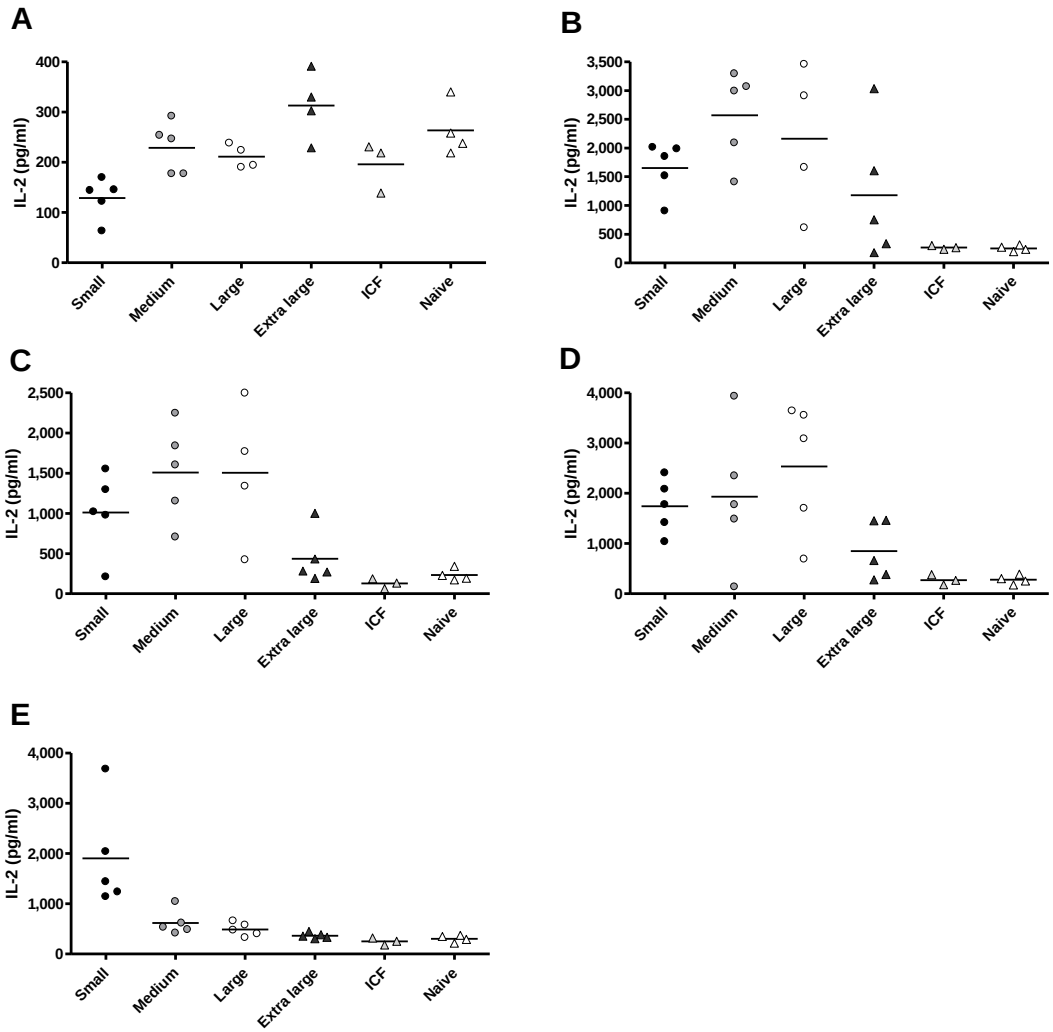

Figure 6.15 IL-2 cytokine production from culture splenocytes derived from unimmunised mice (naïve) or those immunised with Ag85B-ESAT-6 combined with Incomplete Freund's adjuvant (ICF) or DDA:TDB liposomes produced to various vesicle sizes (small, medium, large or extra large. Mice received 3 injections with 2-week intervals and splenocytes were obtained 3 weeks post the final immunisation. Splenocytes were restimulated for 48 hrs in the presence of media alone (A), Ag85B-ESAT-6 at 0.05 µg/ml (B), 0.5 µg/ml (C) or 5 µg/ml (D), or in the presence of 5 µg/ml ConA (E) to act as a positive control. Cytokines were detected from splenocyte supernatants and measured using sandwich ELISAs.

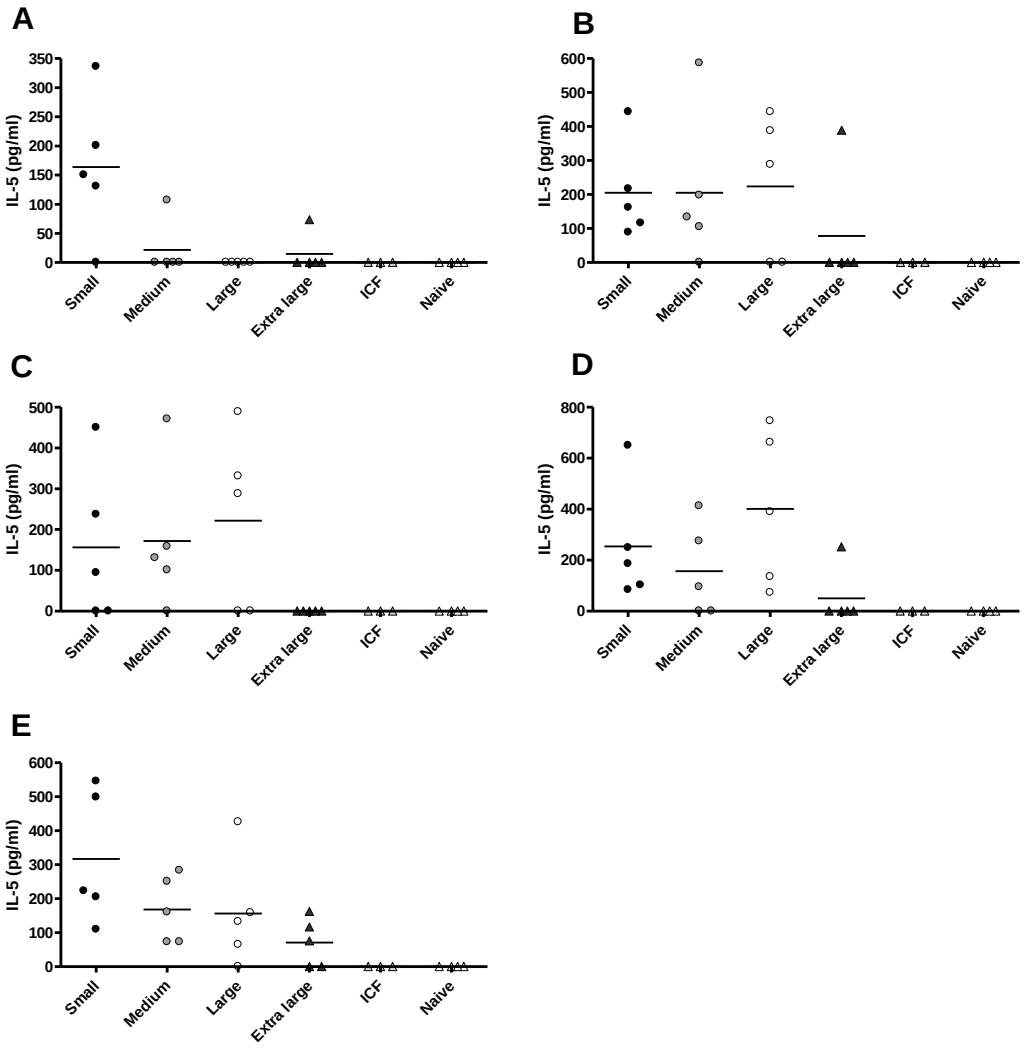

Figure 6.16 Legend as for Figure 6.15 but for detection of IL-5 cytokine.

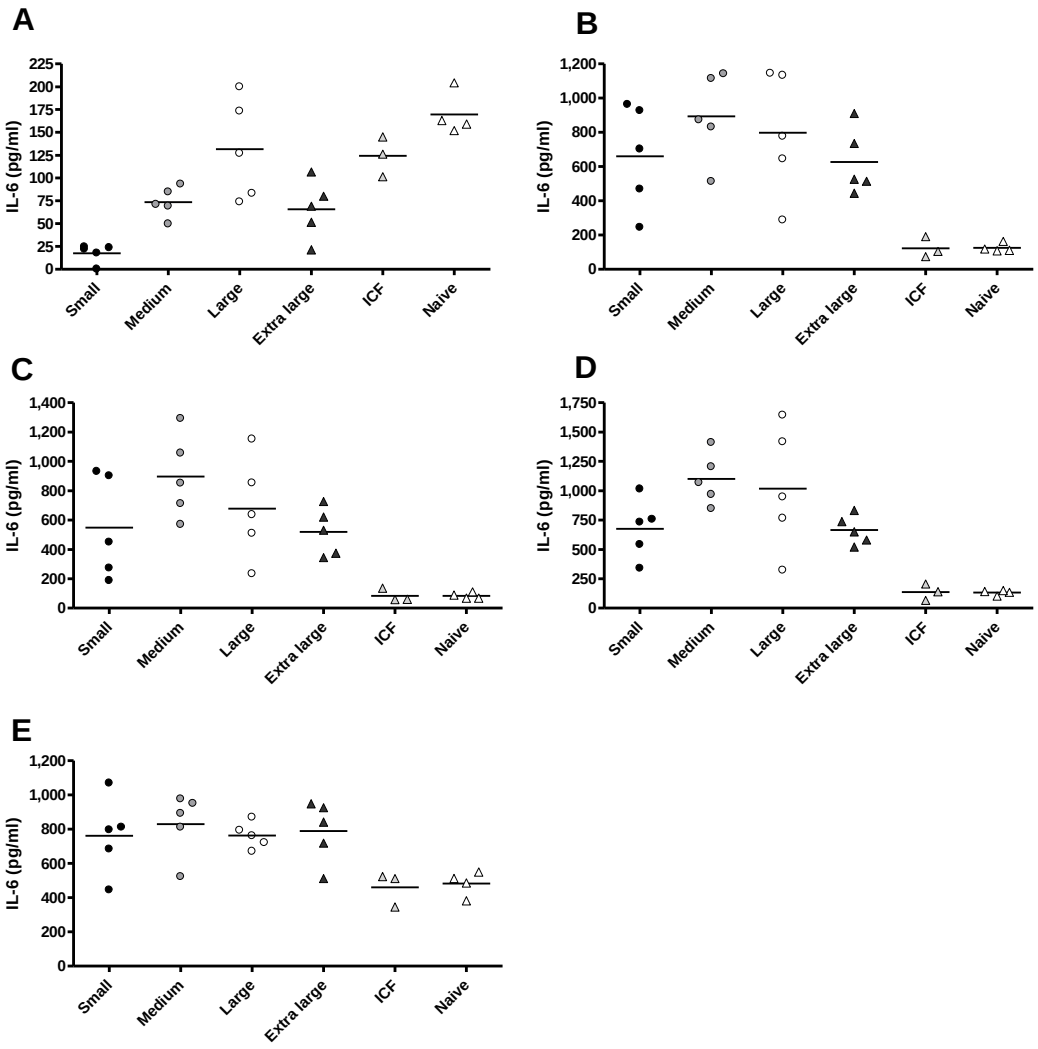

Figure 6.17 Legend as for Figure 6.15 but for detection of IL-6 cytokine.

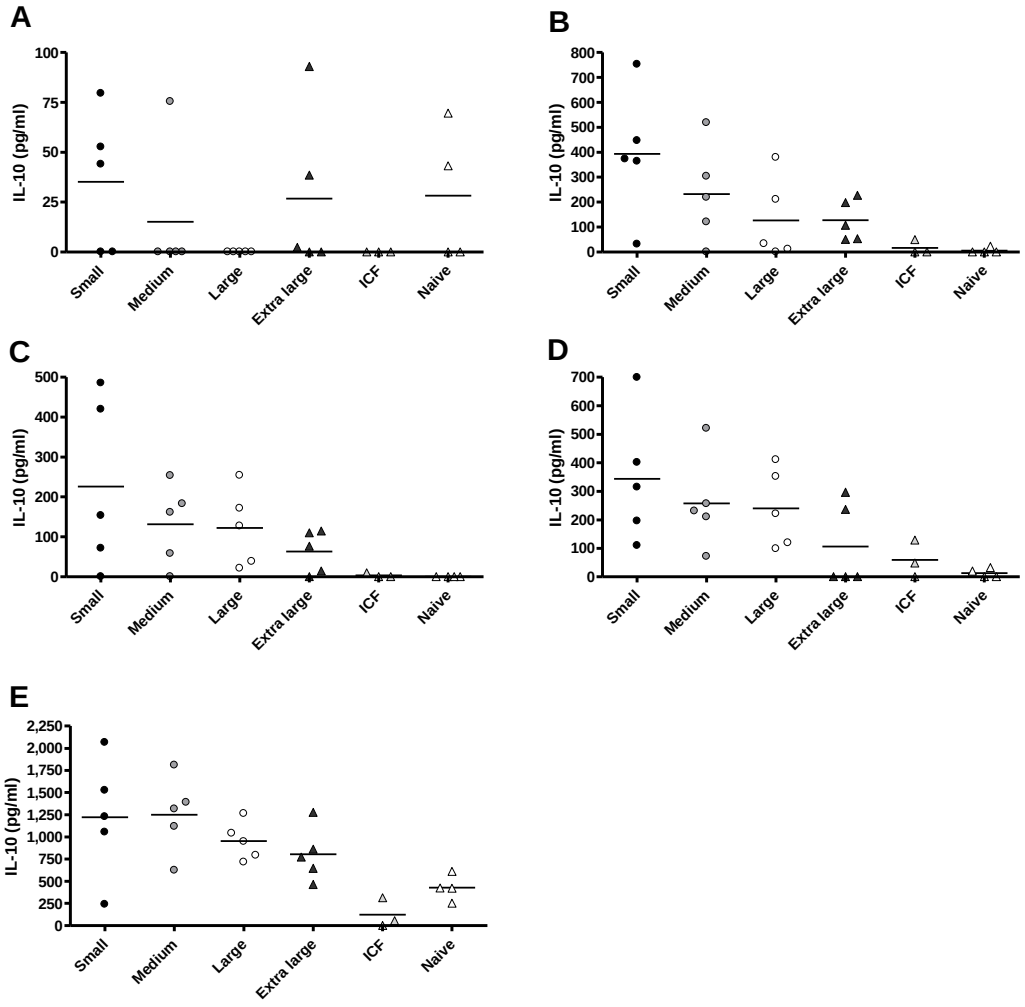

Figure 6.18 Legend as for Figure 6.15 but for detection of IL-10 cytokine.

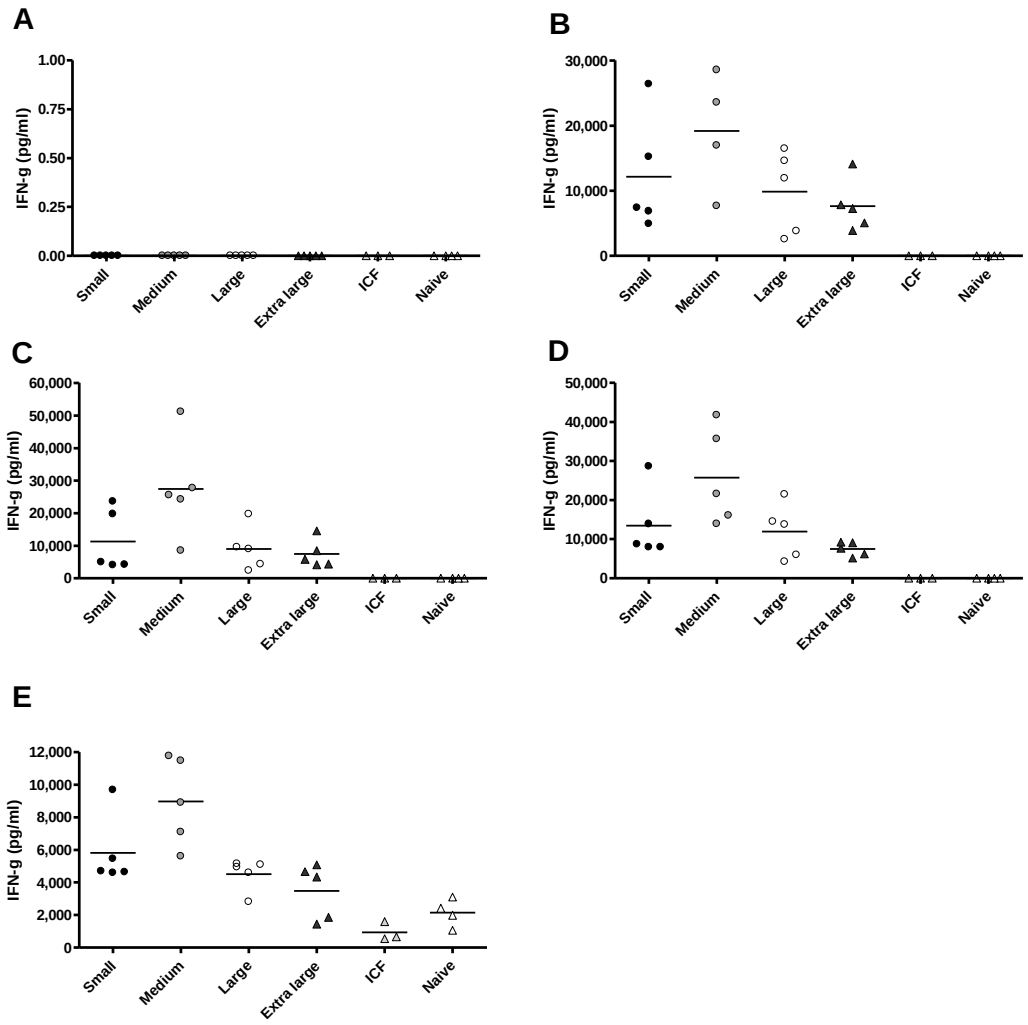

Figure 6.19 Legend as for Figure 6.15 but for detection of IFN-γ cytokine.

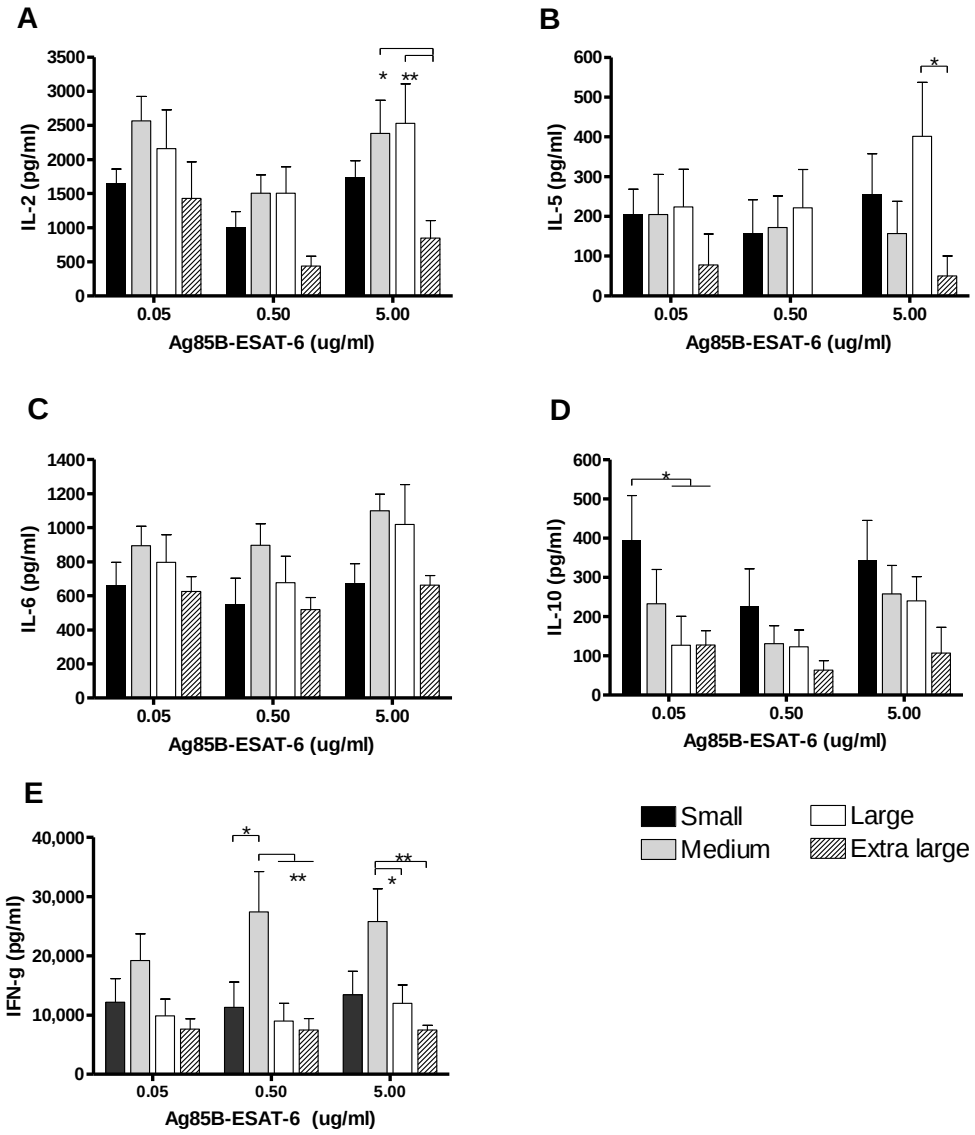

Figure 6.20 Conclusion graphs showing IL-2 (A), IL-5 (B), IL-6 (C), IL-10 (D) and IFN-γ (E) production from splenocytes restimulated with Ag85B-ESAT-6 at 0.05, 0.5 or 5 µg/ml. Results are shown for mice immunised with small, medium, large or extra large DDA:TDB liposomes using the same experimental method described for Figure 6.15.

6.4 Discussion and Conclusion

The role of liposome size remains undetermined in the ensuing immune responses upon liposome adjuvanted immunisation. Although there are reports showing that large liposomes are superior to small liposomes for induction of Th1 responses (Brewer et al., 1998; Mann et al., 2009), the wide range of conflicting data with regards to liposomes in general merited an investigation into the role of DDA:TDB liposome size in the current TB model using Ag85B-ESAT-6. In addition to investigating whether liposome size altered the

kinetics of vaccine movement upon i.m injection, the resulting humoral and cell-mediated immune responses in mice were investigated.

The results indicate that altering the size of DDA:TDB liposomes does indeed affect the biodistribution and immune responses in mice immunised with Ag85B-ESAT-6. Significant differences observed must be solely a property of the vesicle size as this was the only characteristic altered in the liposomal vaccines investigated. The only potential exception to this relates to the extra large liposomes which were formed using PBS buffer as opposed to Tris buffer to rehydrate the lipid films; therefore significant differences in the immune response relating to this formulation may be argued to be salt-induced as opposed to solely size induced. Regardless, the choice of production methods to form DDA:TDB liposomes did indeed result in four significant different sized vesicles, ranging from 200 nm to over 3 µm, which all expressed a cationic surface charge. Levels of Ag85B-ESAT-6 adsorption were high (~ 96 %) due to electrostatic interactions between the liposomes and antigen. Although extra large PBS formed liposomes were not investigated *in vitro*, results relating to small and large DDA:TDB liposomes (medium DDA:TDB liposomes were described in Chapter 3) showed no overt cytotoxicity when applied at 5 µg/ml and liposome endocytosis by cells was observed.

The ability of DDA:TDB liposomes to cause an antigen depot-effect was again observed regardless of whether small, medium or large liposomes were used as the Ag85B-ESAT-6 delivery vehicle. No major differences were noted in liposome draining from the SOI suggesting that the liposome depot effect is more significantly governed by liposome charge and rigidity rather than draining of different vesicle sizes. In correlation with previous experiments (Chapters 3, 4 and 5), the local popliteal lymph node showed higher levels of liposomes than antigen. The very interesting finding that smaller liposomes drained slower to the lymph node than larger liposomes was observed at two early time-points (6 hrs and 24 hrs p.i). This further supports the observation that liposome movement from the injection site is not due to vesicle size related draining from the tissue (otherwise smaller liposomes would be expected in the lymph node quicker). As DDA:TDB liposomes are immunostimulatory due to the inclusion of the mycobacterial glycolipid TDB, faster draining and increased presence of large DDA:TDB liposomes in the lymph node may result in heightened immune responses to simultaneously administered Ag85B-ESAT-6. However only two time-points were studied in this experiment and previous results have show rapid liposome draining to the lymph node to be disadvantageous for

the formation of immune responses (DSPC:TDB and DOTAP:TDB liposomes, Chapters 4 and 5 respectively). Consequently it is also possible that this faster draining of large DDA:TDB liposomes may suggest an earlier peak and subsequent decrease in liposomes presence within the lymph node.

The ability of Ag85B-ESAT-6 adsorbing small, medium, large and extra large DDA:TDB liposomes to induce humoral immunity characterised by IgG antibodies was investigated and compared to the response given upon injection of Incomplete Freund's adjuvant (ICF) in combination with Ag85B-ESAT-6. Unfortunately no significant differences were detected between immunisation groups, however, IgG, IgG1 and IgG2 antibody titres for liposome adjuvanted Ag85B-ESAT-6 vaccines were ≥ ICF antibody titres. In addition to investigating the humoral immune response, the cell-mediated response was measured by detecting levels of splenocyte proliferation and cytokine production upon restimulation with Ag85B-ESAT-6 antigen. Levels of splenocyte proliferation were highly dependent on both the size of the liposome and the concentration of Ag85B-ESAT-6 used to restimulated cells. Increases in vesicle size and Ag85B-ESAT-6 concentration caused significant increases in splenocyte proliferation. These results provide strong reasoning to suggest that larger liposomes are better at presenting antigen to APCs and inducing memory T cell formation. The results relating to cytokine production from the same restimulated splenocytes only show a liposome size mediated effect in the production of IL-10 from restimulated splenocytes, however, IL-10 production is an inverse relationship with splenocyte proliferation. As IL-10 is noted to have inhibitory actions on splenocyte proliferation (Mocellin et al., 2004), it is possible that the poorer proliferative ability of splenocytes derived from small liposome immunised mice is due to IL-10 inhibition. This is also supported by the finding that the highest level of IL-10 production corresponds with the lowest level of splenocyte proliferation, regardless of the liposome size or Ag85B-ESAT-6 restimulation concentration. With the exception of IL-10, there were no other notable results relating to liposome size mediated cytokine production. In correlation with numerous other immunisation studies using DDA:TDB liposomes combined with Ag85B-ESAT-6 (Agger et al., 2008b; Davidsen et al., 2005; Linderstrøm et al., 2009), levels of IFN-γ were high and similar levels were produced regardless of the concentration of Ag85B-ESAT-6 applied to splenocytes for restimulation. Splenocytes therefore respond to this antigenic signal to a high affinity as the difference in the amount of Ag85B-ESAT-6 applied to the cells was 100-fold. In addition to the characteristic Th1 cytokine IFN-γ, IL-2 was also produced to relatively high levels (1000 – 3000 pg/ml) and the combined presence of

these cytokines suggests the induction of central memory T cells which posses multifunctional abilities (Seder et al., 2008).

It is also pleasing to see that none of the liposomal formulations induced high levels of IL-5 which is often measured as the predominant Th2 mediating cytokine and is present in high amounts upon immunisation with aluminium mineral salts. The differences in liposome size in this study do therefore not bias the immune response in a Th1/Th2 way, as measured by IgG1/IgG2 antibody titres and IFN-γ and IL-5 cytokine production. However there does appear to be an increased ability of larger liposomes to induce memory T cells (as noted in proliferation studies) although this may also be due to inhibitory actions of IL-10 limiting proliferation in splenocyte samples collected from mice immunised with smaller liposomes. IL-10, expressed constitutively by a range of cells including mononuclear phagocytes, natural killer cells and $CD4^+$ T cells, is able to affect activation and proliferation of T cells via a number of mechanisms (all reviewed in detail by Moore et al. (Moore et al., 2001)). Firstly IL-10 has been shown to inhibit transport of antigen loaded MHC Cl II molecules to the cell surface thereby limiting the number of TCRs that can become activated. Furthermore IL-10 can down-regulate expression of co-stimulatory molecules CD45, CD80 and CD86 which further limits the ability of successful T cell activation to occur. Finally IL-10 indirectly blocks the co-stimulatory signal CD80/86 (on APCs) with CD28 (on T cells) thereby inhibiting T cell activation (Akdis and Blaser, 2001). However, with regards to this last mechanism, inhibition of T cell activation is only noted for T cells which require the CD28 co-stimulatory signal and therefore implies that T cells which have already experienced antigen (i.e. memory T cells) are not affected by IL-10 as CD28 co-stimulation is not required (Mocellin et al., 2004). These combined actions suggest a targeted role of IL-10 towards naïve T cells or those which are derived from immunised mice in which antigen has been poorly presented and consequently contain a poor memory T cell population. Collectively this suggests that smaller DDA:TDB liposomes are less efficient at Ag85B-ESAT-6 presentation to APCs than larger DDA:TDB liposomes.

Chapter 7: General Discussion

Cationic lipids such as DDA have been known to have immunostimulatory actions for over 40 years (Gall, 1966). However, it is only within the last 5 - 10 years that the molecular mechanisms by which liposomes composed of cationic lipids act are starting to be understood. Liposomes composed of DDA in combination with the highly immunomodulatory glycolipid TDB are in Phase I trials as vaccine adjuvants designed to improve immune responses towards TB vaccination. Of the various methods by which adjuvants are proposed to improve immune responses towards simultaneously administered antigen (Schijns, 2000), DDA:TDB liposomes have been proposed to fulfil at least three mechanisms including improved antigen delivery to APCs, induction of an antigen depot-effect at the SOI and local tissue damage upon injection leading to release of endogenous DAMPS. More recently the involvement of a fourth mechanism involving exogenous PAMPs has been identified; TDB has been shown to bind to the cellular receptor Mincle initiating a series of intracellular cascades resulting in innate immune activation with resultant Th1/Th17 immune responses (Ishikawa et al., 2009; Schoenen et al., 2010; Werninghaus et al., 2009).

The duration of antigen exposure to the host is known to have an effect on the efficiency of the resulting immune response (Obst et al., 2007). As subunit vaccine antigens are generally small inert peptides or proteins that do not contain pathogenic stimuli, their administration without any carrier molecule leads to their rapid removal by the host and therefore little, if any, immune response is mounted. In order to increase the duration of antigen presentation to APCs and subsequently T cells for the initiation of memory responses, liposomes can be used to simultaneously delivery antigen. Whilst liposomes composed of DDA have been noted to cause an antigen depot-effect at the SOI (Holten-Andersen et al., 2004), this has not been quantified, nor has its role on the immunogenicity of vaccines composed of DDA been established. Therefore, the general aim of this thesis was to investigate which physicochemical parameters of DDA-based liposomes lead to the desirable immune responses noted in immunisation studies. By altering parameters such as liposomal charge and size, membrane fluidity and stability, or the direct substitution of DDA with other lipids with documented immunostimulatory actions, the properties which define DDA-based liposomes as successful adjuvants were consequently investigated.

7.1 DDA:TDB Liposomes as a Mediator of the Antigen Depot-effect and Long Term Immune Responses

In correlation with described observations of DDA:TDB liposome deposition at the SOI (Holten-Andersen et al., 2004), we have shown and indeed quantified the DDA:TDB depot-effect and note that even after 2 weeks p.i more than one third of the administered liposome dose can be recovered from the SOI (Henriksen-Lacey et al., 2009). Furthermore, significant antigen retention at the SOI was observed, however, this was highly dependent on antigen adsorption to the DDA:TDB liposomes as a model antigen which showed poor adsorption to DDA:TDB liposomes was not efficiently retained at the SOI (Henriksen-Lacey et al., 2010c). Addition of stabilising cholesterol to DDA:TDB liposomes had little effect on either liposome or antigen movement from the SOI, suggesting that whilst cholesterol may be required to improve liposome stability thereb extending the shelf-life, upon *in vivo* administration little change in the physicochemical nature of the vesicles is observed. Although not reported here, the inclusion of cholesterol in DDA:TDB liposomes has been shown to decrease the IFN-γ response (Christensen et al., 2009), suggesting that cholesterol has an effect on the cell-mediated immune response.

The liposome and antigen depot-effect noted upon injection of DDA:TDB liposomes adsorbing Ag85B-ESAT-6 antigen was not limited to i.m injection as similarly strong depots were observed after s.c injection (Henriksen-Lacey et al., 2009). In addition, immunogenicity studies showed little difference in the cell-mediated immune responses when DDA:TDB liposomes were administered via the i.m, s.c or i.d routes of injection (Mohanan et al., 2010). Therefore the depot-effect does not appear to be tissue specific although local cell populations and proximity to local lymph nodes may have an effect on the immune responses noted.

The ability of DDA:TDB liposomes to induce long term immunity in mice has recently been described by Lindenstrøm and colleagues, again using the TB model antigen Ag85B-ESAT-6 (Linderstrøm et al., 2009). Their findings also highlight the Th1 polarising nature of DDA:TDB liposomes and their ability to induce production of multifunctional memory T cells which are vital against secondary TB infection. Such T cells isolated > 1 year after immunisation retained their proliferative ability (divisional abilities were ~ 10 times higher than non-vaccinated controls), and mice did not succumb to disease in *Mycobacterium tuberculosis* challenge experiments (Linderstrøm et al., 2009). These results show the strong ability of cationic DDA:TDB liposomes to act as an adjuvant for Ag85B-ESAT-6

antigen and the authors raised the importance of long-term slow release of antigen after administration. It is mutually accepted that antigen dose and drainage kinetics are important factors in the resulting immune response. However, whilst some argue for escalating doses (Johansen et al., 2008), it is also clear that single or multiple administration of depot-forming adjuvants is able to induce the desired response (Linderstrøm et al., 2009).

7.2 The Role of Liposome Charge

Liposomes expressing a cationic surface charge aggregate with serum proteins and consequently, upon *in vivo* injection, it was hypothesised that the strong retention of DDA:TDB liposomes at the injection site may have been due to serum protein-mediated liposome aggregation. This was also the basis for the hypothesis that simultaneously administered antigen, regardless of its adsorption efficiency to DDA:TDB liposomes, is also retained at the SOI due to cationic liposomal aggregation. To determine the role of liposome charge in the liposome and antigen deposition noted at the SOI, the cationic lipid DDA was substituted with DSPC therefore donating a neutral/slightly anionic surface charge to the liposomes due to the zwitterionic nature of DSPC. As hypothesised, DSPC:TDB liposomes exhibited significantly faster rates of removal from the SOI, as did simultaneously administered (although not adsorbed due to lack of electrostatic interactions) Ag85B-ESAT-6 antigen. This corresponded with a poor ability of neutral DSPC:TDB liposomes to induce T cell proliferation, cause IFN-γ production and induce production of T cells with memory ability (Henriksen-Lacey et al., 2010c). *In vitro* studies comparing DDA:TDB and DSPC:TDB liposomes highlighted the importance of liposomal surface charge for cellular uptake and activation of DCs to induce Il-1β production; whilst DDA:TDB liposomes efficiently associated (both uptaken and adsorbed) to macrophages, the lipid concentration administered was vital as toxicity was observed. The results consequently suggest that IL-1β production by DCs may well be due to DC uptake of DDA:TDB liposomes but not DSPC:TDB liposomes. Whilst this feature had no relation to the depot-effect (as studies were conducted *in vitro* using microplates), it does suggest that if even if exposure of DSPC:TDB liposomes to APCs was prolonged, no improvements in liposome uptake would be observed. The cationic charge of DDA:TDB liposomes therefore prolongs its retention at the SOI as well as increasing its uptake by APCs.

7.3 The Importance of Antigen Choice for the Depot-effect and Eliciting Immune Responses

Numerous immunisation studies use model antigens such as OVA to study humoral and cell-mediated immunity. The reasons for this are numerous; OVA is inexpensive and readily available, there is a lot of literature regarding its use, and there are many research techniques available such as transgenic mice or fluorescently labelled OVA. However we have shown that the immune response derived from Balb/c mice immunised with DDA:TDB liposomes and OVA is not typical of the strong Th1 polarising nature of DDA:TDB liposomes observed in other immunisation studies (Christensen et al., 2010a; Davidsen et al., 2005; Holten-Andersen et al., 2004; Vangala et al., 2007). When Balb/c mice were immunised with DDA:TDB liposomes adsorbing OVA, no detectable IgG2 response was observed and splenocytes were extremely poor at cytokine production in response to ConA or restimulation with OVA. Similar poor cell-mediated immune responses were observed in a collaborative study conducted at University Hospital of Zurich (Mohanan et al., 2010). As explained later (section 7.6), substitution of Balb/c mice for the more Th1 polarising mouse strain C57Bl/6 (Charles et al., 1999), in addition to substituting OVA for the immunogenic antigen Ag85B-ESAT-6 (Olsen et al., 2001), re-established the strong Th1 immune responses typically elicited by DDA:TDB liposomes adsorbing Ag85B-ESAT-6.

The role of antigen was also investigated in biodistribution studies whereby the importance of antigen adsorption to DDA:TDB liposomes was questioned. DDA:TDB liposomes adsorb strongly to Ag85B-ESAT-6 and initiate high levels of Ag85B-ESAT-6 retention at the injection site; would antigens which displayed poor adsorption abilities to DDA:TDB liposomes also be well retained? To answer this, two antigens were chosen which expressed medium and poor adsorption abilities to DDA:TDB liposomes. Whilst simultaneous administration of antigen expressing medium adsorption abilities to DDA:TDB liposomes was retained at the SOI to the same efficiency as Ag85B-ESAT-6, the poorly adsorbing antigen showed a similar drainage profile to antigens administered without a delivery system (Henriksen-Lacey et al., 2010c). Therefore a degree of antigen-liposome association was required although the importance of this was not translated into immunisation studies. Regardless, the role of antigen in establishing immune responses but also prolonging its own exposure to APCs is significant and should be considered when designing costly *in vivo* experiments.

7.4 Comparison of Fluid Verses Rigid Liposomes as Effective Adjuvants

As it was established that cationic charge and antigen adsorption were both important factors for a liposome and antigen depot-effect at the SOI, the role of liposomal membrane fluidity was consequently investigated. Membrane fluidity can be altered by the inclusion of unsaturated hydrocarbon chains into the lipid, thereby altering the VDW forces between adjacent lipid monomers and disrupting the bilayer organisation. A direct comparison against DDA:TDB liposomes was made using DODA which is an unsaturated analogue of DDA and is therefore hypothesised to contain fluid bilayers at physiological temperatures. The increase in bilayer fluidity noted with DODA-based liposomes caused faster liposome drainage from the SOI and a subsequent faster appearance of liposomes in the local lymph nodes. However, this did not correlate with improved immune responses as mice immunised with DODA:TDB liposomes produced little IFN-γ and IL-17 from restimulated splenocytes. Furthermore, the T cells derived from these mice displayed low levels of memory T cells. Analysis of DCs from DODA:TDB liposome injected mice also showed a significantly lower uptake of DODA:TDB liposomes and reduced abilities to stimulate upregulation of co-stimulatory markers when compared to DDA:TDB liposome injected mice (Christensen et al., 2010b). Liposome rigidity therefore plays an important role in prolonging cellular exposure to liposomes which has significant effects on APC activation and long term memory induction.

7.5 Choosing the Correct Immunostimulatory Cationic Lipid

Whilst DDA-based liposomes express numerous characteristics which make them efficient adjuvants for subunit vaccines, it is interesting to note that no direct immunostimulatory actions have been attributed to the lipid DDA (Korsholm et al., 2006; Vangasseri et al., 2006). In contrast, cationic lipids such as diC14-amidine, DOTAP and DC-Chol have been shown to have immunomodulatory properties including upregulation of co-stimulatory molecules and chemokine induction (Cremel et al., 2006; Tanaka et al., 2008; Vangasseri et al., 2006; Yan et al., 2007). DOTAP and DC-Chol are cationic liposome forming lipids and therefore it was presumed that they would also express an ability to form a liposome depot at the SOI. However, our results showed that although DC-Chol-based liposomes did indeed form a liposome depot similar to that noted by DDA, DOTAP-based liposomes

drained rapidly from the SOI. In fact, DOTAP:TDB liposomes expressed a similar liposome and antigen depot-effect to DODA:TDB liposomes, presumably due to the unsaturated nature of both lipids and therefore a similar bilayer fluidity *in vivo*. DC-Chol-based liposomes had a similar biodistribution to DDA:TDB liposomes which suggests a rigid nature to the vesicles. The results relating to the immune responses of these liposomal formulations were interesting as the previously documented immunostimulatory abilities of DOTAP-based liposomes did not translate to increased levels of IFN-γ in immunisation studies. Furthermore, whilst DC-Chol:TDB liposomes were strong inducers of early IFN-γ production, they failed to maintain this for a longer period (Henriksen-Lacey et al., 2010b). A study is currently ongoing to investigate the functionality of T cells derived from mice immunised with DOTAP:TDB and DC-Chol:TDB liposomes (personal communication, Dennis Christensen). Therefore it appears that whilst DOTAP-based liposomes are efficient stimulators of DCs, they are poor at antigen presentation and activation of cell-mediated immune responses. The opposite was noted for DDA:TDB liposomes.

7.6 Investigating the Role of Vesicle Size

Throughout we have highlighted the strong immunogenic properties of DDA:TDB liposomes which make these vesicles suitable adjuvants for Ag85B-ESAT-6 antigen delivery. As a final investigation, the importance of vesicle size was analysed to determine whether changes in DDA:TDB vesicle size altered their ability to cause a liposome and antigen depot-effect, and successfully immunise mice with Ag85B-ESAT-6. Whilst no differences in the depot-effect were noted across the size ranges studied, significant size-related differences in liposome draining to the local lymph nodes, splenocyte proliferation and IL-10 cytokine production from restimulated splenocytes were noted. Larger liposomes drained faster to the lymph node and splenocytes derived from mice immunised with larger liposomes proliferated to higher levels upon restimulation *ex vivo*. By contrast, the smallest liposomes of ~ 200 nm showed a slower drainage profile to the lymph node and, upon restimulation of splenocytes derived from small liposome immunised mice, lower levels of splenocyte proliferation were noted. Liposomes of all size ranges elicited similar levels of IgG (IgG1 and IgG2) antibodies and IFN-γ, indicative of successful humoral and cell-mediated immune responses respectively. Whilst these results therefore suggest an improved ability of larger liposomes to present antigen to APCs which in turn can stimulate improved memory T cell, it was interesting to note that

restimulation of splenocytes derived from mice immunised with small DDA:TDB liposomes resulted in elevated levels of IL-10. In fact, there was an inverse correlation between IL-10 production and splenocyte proliferation. As IL-10 is documented to inhibit cell proliferation (Mocellin et al., 2004; Moore et al., 2001), it remains possible that splenocytes derived from small DDA:TDB liposome immunised mice show increased production of IL-10 which in turn inhibits their own proliferation. Alternatively the opposite may occur in which splenocytes derived from larger DDA:TDB liposome immunised mice produce an IL-10 receptor agonist which blocks IL-10 mediated inhibition of proliferation as well as autocrine IL-10 production.

7.7 Conclusions and Further Thoughts

It is evident that cationic liposomes have a special ability to improve immune responses towards simultaneously administered antigens. Whilst a model to define the required characteristics of a successful cationic liposomal adjuvant has not been finalised in these characterisation studies, we have identified a number of physicochemical properties which appear to improve immune responses to the vaccine antigen. The ideal liposome in this respect appears to have:

- a cationic surface charge that shows strong adsorption to the vaccine antigen
- exhibits a rigid bilayer
- is large (> 1 or 2 μm) in size

This combination leads to a strong liposome and antigen depot-effect, efficient liposome and antigen draining to the local lymph node, uptake and activation by DCs, strong humoral immune responses with IgG2 antibody isotypes, IFN-γ production and most importantly, long lasting immune responses which are characterised by memory T cells. Throughout the studies DDA:TDB liposomes were superior to other liposome formulations in their abilities to immunise mice with Ag85B-ESAT-6. The results retaining to DDA:TDB vesicle size illustrate an interesting finding as the immunogenicity of 'normal' DDA:TDB liposomes appears to be augmented upon increasing their vesicle size. These results merit further characterisation of the immune responses elicited by the larger version of DDA:TDB liposomes. Experiments such as determination of the route of endocytosis would be of interest as vesicle size-dependent internalisation is well documented (Brewer et al.,

2004; Brewer et al., 1998; Mann et al., 2009). Furthermore, determination of the role played by IL-10, if any, in splenocyte proliferation could be of interest for therapeutic vaccines. The detection of the pro-inflammatory cytokine IL-1β from DCs stimulated with DDA:TDB liposomes also raises the question of whether DDA:TDB liposomes and adjuvants such as aluminium salts share more features than originally though. Aluminium salts are known to cause an antigen depot-effect and stimulate the inflammasome complex which is involved in IL-1β production. DDA:TDB liposomes also cause an antigen-depot effect and stimulate IL-1β production. Consequently, are some of the adjuvantal effects noted by DDA:TDB liposomes a result of local tissue damage causing release and production of alarmins? Full production of active IL-1β also requires TLR signalling (Marrack et al., 2009; Monie et al., 2009) and although no binding of DDA:TDB liposomes to TLRs has yet been discovered, TDB is known to bind to the C-type lectin Mincle (Ishikawa et al., 2009; Schoenen et al., 2010). Therefore perhaps there is a potential role for Mincle to stimulate the inflammasome? A further interesting correlation between DDA liposomes and aluminium salts is their shared inability to stimulate upregulation of co-stimulatory molecules on DCs (Korsholm et al., 2006; Sun et al., 2003), however incorporation of TDB to DDA liposomes can overcome this problem.

To finalise, cationic liposomes remain a complex system whereby small changes in the physicochemical characteristics result in large changes in the observed immune responses towards vaccine antigens. The results presented in this thesis come at an interesting time as DDA:TDB liposomes are in clinical trials. Hopefully the results presented in this thesis will expand our understanding of how DDA:TDB liposomes work.

8. References

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The use of cationic liposomes as adjuvants for subunit protein vaccination, M.Henriksen-Lacey, V.W.Bramwell, D.Christensen, E.M.Agger, P.Andersen and Y. Perrie, UKPharmSci, Nottingham University, UK, September 2010

Immunostimulatory Cationic Liposomes as Adjuvants in a Novel TB Vaccine, M. Henriksen-Lacey, V. W. Bramwell, D. Christensen, E.M. Agger, P. Andersen and Y. Perrie, Controlled Release Society (CRS) Annual Meeting 2010, Portland, USA, August 2010

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